Proc. Natl. Acad. Sci. USA
Vol. 73, No. 12, pp. 4304–4308, December 1976
Biochemistry

Segment-long-spacing aggregates and isolation of COOH-terminal peptides from type I procollagen

(collagen precursor/interchain disulfide bonds/electron microscopy)

HANS-PETER HOFFMANN, BJØRN R. OLSEN, HAN-TEE CHEN, AND DARWIN J. PROCKOP

Department of Biochemistry, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, N.J. 08854

Communicated by George E. Palade, June 29, 1976

ABSTRACT **Type I procollagen secreted by matrix-free cells from chick embryo tendons was purified by DEAE-cellulose chromatography. Electron microscopy of segment-long-spacing aggregates of the procollagen demonstrated the presence of both NH_2-terminal and COOH-terminal extensions not found in collagen. The procollagen was digested with bacterial collagenase and the COOH-terminal fragments were isolated by gel filtration and polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Analysis of tryptic peptides demonstrated that the COOH-terminal extensions on the proα1 and proα2 chains had different primary structures.**

Procollagen, the precursor of collagen, is larger than collagen because the three polypeptide chains in the molecule contain peptide extensions (for reviews, see refs. 1–4). Initial electron microscopic studies demonstrated that the NH_2-terminal ends of the molecule contained extensions (5–7), and from data available at the time it was assumed that the COOH-terminal ends were about the same as the COOH-terminal ends of collagen. Subsequently, Tanzer *et al.* (8) suggested that peptide extensions were present at both the NH_2- and COOH-terminal ends of procollagen, and this suggestion has recently been confirmed by chemical studies on Type I (9–11) and Type II (11) procollagen from several sources.

METHODS

Partial Purification of Tendon Procollagen. Cells were prepared by enzymic digestion of tendons from 17-day-old chick embryos (12, 13). About 2×10^9 cells were incubated at a concentration of 7.5×10^6 cells per ml at 37° for 2 or 4 hr with 100 μCi of either [^{14}C]proline or a mixture of ^{14}C-labeled amino acids (New England Nuclear) in Krebs medium. The medium was removed by centrifugation at 25° (12), protease inhibitors (14, 15) were added (11), and protein in the medium was precipitated with 176 mg/ml of ammonium sulfate (Baker). The precipitate was dissolved at 4° in 1/50 the original medium volume of 0.4 M NaCl in 0.1 M Tris·HCl buffer, pH 7.8, and frozen. About 25 ml of the sample was dialyzed at 4° against 2 M urea and 0.1 M Tris·HCl buffer, pH 7.8, and chromatographed (16) on a 1.6×14 cm column of DEAE-cellulose (Whatman DE-52). The column was eluted at 4° and at a flow rate of 78 ml/hr with a linear gradient prepared with 200 ml of 2 M urea and 0.1 M Tris·HCl and 200 ml of the same buffer containing 0.1 M NaCl. Fractions were 8 ml. The [^{14}C]procollagen was dialyzed overnight at 4° against 0.4 M NaCl and 0.1 M Tris·HCl buffer, pH 7.8. Protein concentration was determined by amino acid analysis.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. This procedure was carried out in slab gels. (17, 18). For examination of procollagen polypeptides a concave gradient gel was prepared with 4.5% polyacrylamide at the top and 22.5% polyacrylamide at the bottom. The concentrations of *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were reduced according to the procedure of O'Farrell (19) and the stacking gel was replaced with a strip of 4.5% separating gel. The slabs were stained with 0.25% Coomassie Blue and 20% trichloroacetic acid for 1–15 hr at room temperature, and then they were destained in 7.5% acetic acid and 15% methanol. For examination of the COOH-terminal peptides, a gel of 15% acrylamide was used (11). For fluorography, the gels were impregnated with the organic scintillator 2,5-diphenyloxazole (PPO) to convert the energy of β-particles to visible light, which forms an image on blue-sensitive x-ray film (20).

Preparation of Segment-Long-Spacing (SLS) Aggregates. [^{14}C]Procollagen in 0.4 M NaCl and 0.1 M Tris·HCl buffer, pH 7.8, was dialyzed overnight at 4° against 0.01 M acetic acid and 0.2% disodium ATP (Sigma). The SLS aggregates were collected on carbon-coated grids, and were stained with either 1% sodium phosphotungstate, pH 7.0, or 1% ammonium molybdate, pH 5.0. The specimens were examined in a JEM-100B electron microscope.

Isolation of COOH-Terminal Peptides. About 2 mg of procollagen purified by DEAE-cellulose chromatography (above) was incubated at 37° for 3 hr with 250 μg of collagenase (12, 21). The digest was chromatographed at 4° on a 1.5×90 cm column of 8% agarose (Bio-Gel, A-1.5m) equilibrated and eluted with 0.1 M Tris·HCl, pH 7.8 (12). The peak containing the disulfide-linked COOH-terminal peptides (11, 12) was then taken for $NaDodSO_4$ gel electrophoresis using 15% polyacrylamide. The gel was stained for 10 min at room temperature with 0.25% Coomassie Blue in 20% trichloroacetic acid, and it was destained for 5 min in 7.5% acetic acid and 15% methanol. Portions of the gel containing the two COOH-terminal peptides were cut out separately and extracted at room temperature in 0.1% $NaDodSO_4$ and 0.01 M NH_4HCO_3 (22). The extracted peptides were precipitated with 10 volumes of cold HCl/acetone (23), the pellets were re-dissolved in 1 ml of 2% $NaDodSO_4$, reduced with 18 mM dithiothreitol, and carboxymethylated with 20 mM iodoacetic acid. The samples were re-precipitated with HCl/acetone and then dissolved in 1 ml of 0.1 M NH_4HCO_3. Tryptic peptides were prepared by digesting the samples with 20 μg of tosylphenylalanylchloromethyl ketone (TPCK)-treated trypsin (Worthington) at 37° for 4 hr. After lyophilization, the peptides were fingerprinted according to the method of Bates *et al.* (24).

RESULTS

Purification of Procollagen. Proteins secreted into the medium by the tendon cells were precipitated with ammonium sulfate and then chromatographed on DEAE-cellulose (Fig. 1).

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; SLS aggregates, segment-long-spacing aggregates.

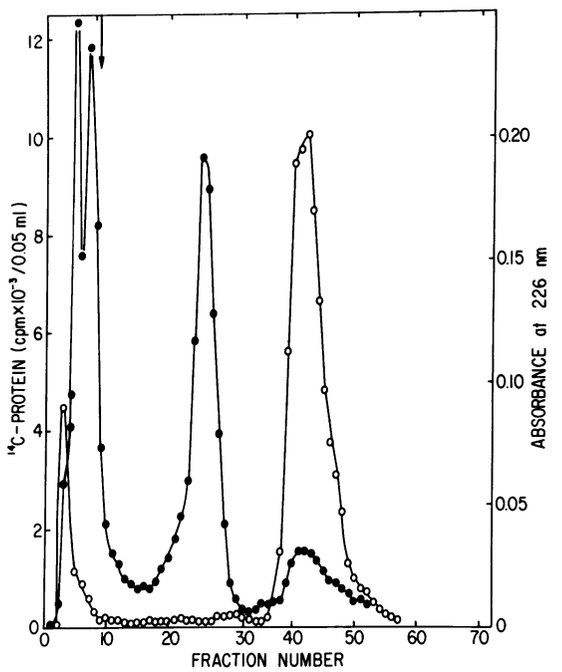


FIG. 1. DEAE-chromatography of [^{14}C]procollagen. Conditions are as described in *text*. ●, Absorbance at 226 nm; ○, ^{14}C-labeled protein. Arrow indicates start of gradient elution.

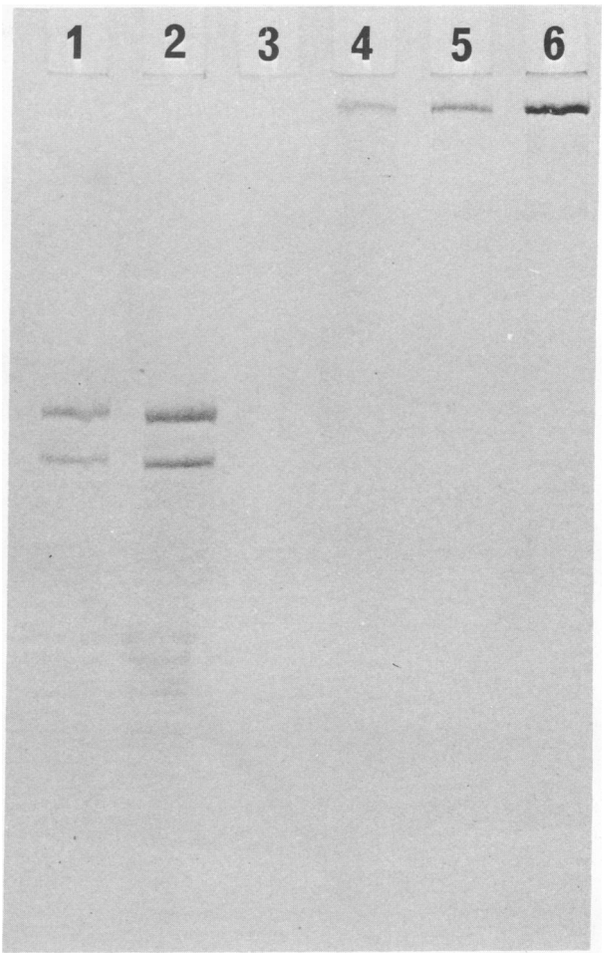


FIG. 2. $NaDodSO_4$ gel electrophoresis of procollagen purified by DEAE-chromatography. Well 1: 7 μg of procollagen, reduced. Well 2: 15 μg of procollagen, reduced. Well 3: blank sample well. Well 4: 7 μg of procollagen treated with 50 mM iodoacetamide to minimize disulfide exchange. Well 5: 7 μg of procollagen. Well 6: 15 μg of procollagen. Migration is toward the anode, at the bottom.

About 25% of the ^{14}C-labeled protein applied to the column was recovered in the break-through volume and about 75% was recovered in a peak in fractions 35–50. Fractions 35–50 also contained a peak of ultraviolet-absorbing material. The major peak of radioactivity (fractions 35–50) was dialyzed and examined by $NaDodSO_4$ gel electrophoresis (Fig. 2). After reduction with 2-mercaptoethanol, most of the protein was recovered in two bands, one in the same electrophoretic position as proα1 chains and the other in the same electrophoretic position as proα2 chains (see ref. 11). A smaller amount of protein of higher mobility was also seen. When the same samples were examined by $NaDodSO_4$ gel electrophoresis without reduction, most of the protein was recovered in a single band of low mobility (Fig. 2). When the gel was examined by fluorography (not shown), essentially all the ^{14}C in the gel was found in the major bands stained by Coomassie Blue.

SLS Aggregates of Procollagen. SLS aggregates were prepared from the procollagen secreted into the medium. The structure of the aggregates was the same when they were prepared from the ammonium sulfate precipitate of the medium and when they were prepared from procollagen purified by DEAE-cellulose chromatography. The NH_2-terminal extensions on the SLS aggregates (Fig. 3) were the same as the NH_2-terminal extensions on partially degraded procollagen observed previously (5). The COOH-terminal ends, however, differed from those of the SLS-aggregates previously obtained from the same source in that they had large additional extensions (Figs. 3 and 4). The presence of the COOH-terminal extensions appeared to spread out the aggregates in a fan-like array, and the structures appeared to adhere to each other at the COOH-terminal ends. The length of the NH_2-terminal extensions was about 130 Å or about the same length as reported previously (5), but it was difficult to estimate the length of the COOH-terminal extensions because of their irregular shape. As indicated (Fig. 4), the NH_2-terminal extensions were missing from many of the SLS aggregates when protease inhibitors were not added before precipitating the medium with ammonium sulfate. The COOH-terminal extensions were, however, largely intact. In previous experiments carried out without protease inhibitors (5), the NH_2-terminal extensions were retained, apparently because the procollagen was not re-precipitated under neutral conditions, but instead the pellet from the first ammonium sulfate precipitation was dispersed in 0.1 M acetic acid. In the same experiments (5), the COOH-terminal extensions were largely (5) but not completely lost (see Fig. 1 in ref. 25), apparently because the procollagen was dialyzed for 24 hr against 0.01 M acetic acid before the SLS aggregates were formed by dialysis against 0.1% disodium ATP in 0.01 M acetic acid.

Isolation of Two Types of COOH-Terminal Extensions. To isolate the COOH-terminal extensions, tendon cells were incubated with a mixture of fifteen ^{14}C-labeled amino acids, the medium procollagen was isolated as described above, and it was digested with bacterial collagenase (12). A disulfide-linked trimer was then isolated by gel filtration of the digest (11, 12). The trimer was previously shown to be derived from the COOH-terminal region of procollagen (11, 12); here the trimer was examined by $NaDodSO_4$ gel electrophoresis (Fig. 5). When examined without reduction with 2-mercaptoethanol, the fragment migrated as a single band with an apparent molecular weight of about 100,000 compared to standards of globular proteins. After reduction, two bands of slightly different

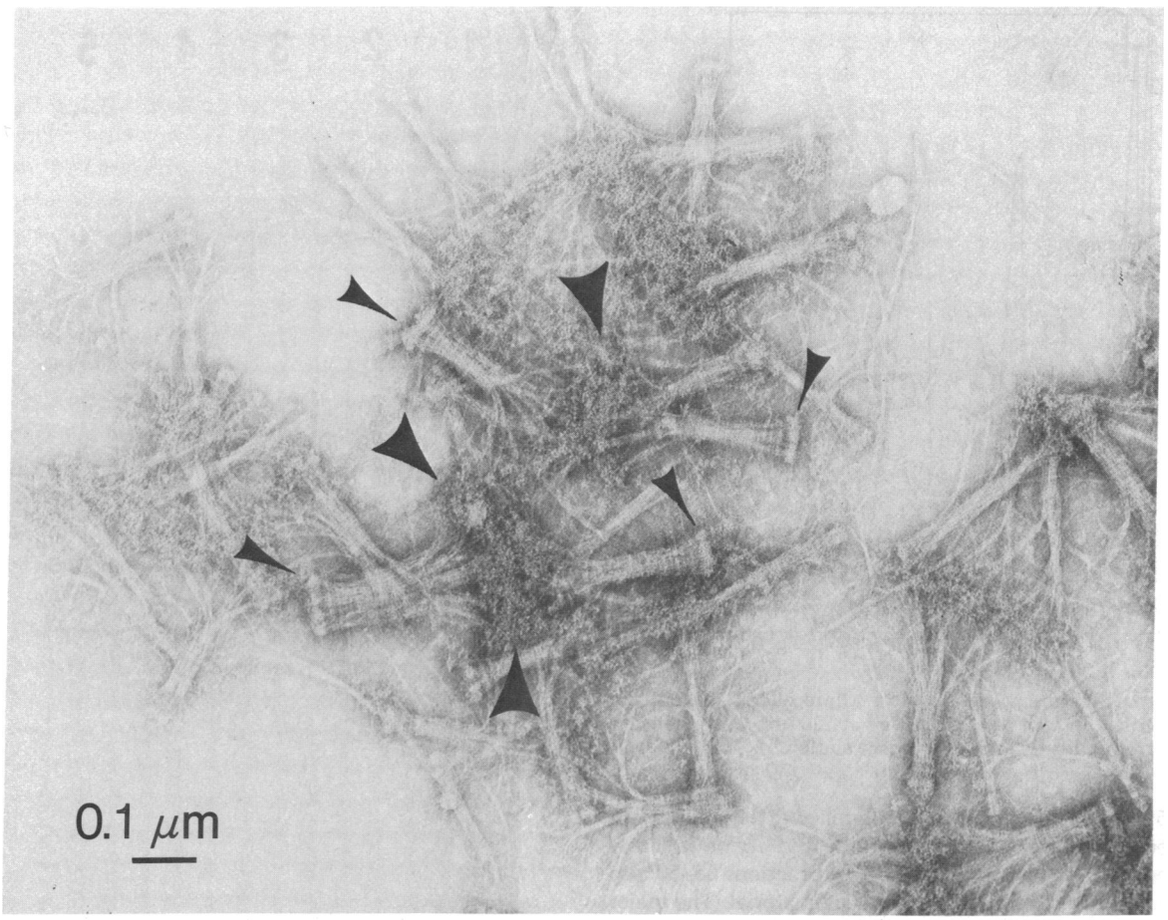


FIG. 3. SLS aggregates of procollagen from the medium of tendon cells. Protease inhibitors were added to medium from tendon cells and the procollagen was precipitated with 176 mg/ml of ammonium sulfate (see *Methods*). The pellet was dialyzed against 0.4 M NaCl and 0.1 M Tris buffer, pH 7.8, and used to prepare SLS aggregates (see *text*) which were negatively stained with 1% sodium phosphotungstate. The NH_2- and COOH-terminal ends of the SLS aggregates were identified by comparing the banding pattern to SLS aggregates of collagen (see ref. 5). Large arrows: COOH-terminal ends of SLS aggregates. Small arrows: NH_2-terminal ends of SLS aggregates. Examination of the SLS aggregates at higher magnification (not shown) indicated that the NH_2-terminal extensions were similar to those seen previously (see Fig. 5 in ref. 5). Magnification ×87,000.

mobilities were observed, and these had apparent molecular weights of about 35,000. In order to estimate the relative amount of peptide in the two bands, the fluorograms were scanned (11) and the optical densities of the two peaks were integrated. The results indicated that the ratio of ^{14}C label in the two bands was 2:1.

The COOH-terminal peptides in the two bands obtained after reduction were eluted from the gels and examined by a microscale fingerprinting technique after cleavage with trypsin (24). A distinct fingerprint was obtained from each COOH-terminal peptide (Fig. 6).

DISCUSSION

As discussed elsewhere (1), matrix-free cells prepared from embryonic tendons offer several advantages over other cell systems for studying the synthesis and secretion of procollagen. The results presented here demonstrate that little purification is necessary to obtain intact procollagen from the medium of this cell system. When a mixture of protease inhibitors (11, 14, 15) was added to the medium at the end of the incubation period, partial degradation of the protein was prevented and most of the ^{14}C-labeled protein recovered from the medium consisted of procollagen containing both NH_2- and COOH-terminal extensions (8–11). Major contaminating proteins were removed by DEAE-cellulose chromatography (16).

The COOH-terminal extensions seen in SLS aggregates of the procollagen were similar but were more readily visualized than the COOH-terminal extensions seen in SLS aggregates that Tanzer *et al.* (8) obtained from a cloned line of fibroblasts and that contained a heterogeneous population of procollagen subunits. The COOH-terminal extensions seen here were large and irregular, and they appeared to prevent close packing of the molecules in the SLS aggregates. In retrospect, it is now apparent that if protease inhibitors are not added to the medium immediately after incubation of the cells, the procollagen secreted by freshly isolated tendon cells readily undergoes partial digestion by endogenous proteases. If the protein is exposed to acidic conditions, the COOH-terminal extensions are more readily lost through proteolysis (5). If the procollagen is processed under neutral conditions, the NH_2-terminal extensions of the molecule are more likely to be lost (11).

After digestion of the purified procollagen with bacterial collagenase, the largest collagenase-resistant fragment in the digest consists of three disulfide-linked peptides which originate from the COOH-terminal region of the molecule (11, 12). The size of the fragment appears to be about the same as that of the fragment recently isolated from chick bone procollagen digested with bacterial collagenase (26). Murphy *et al.* (26) reported on amino acid analysis of the similar fragment from

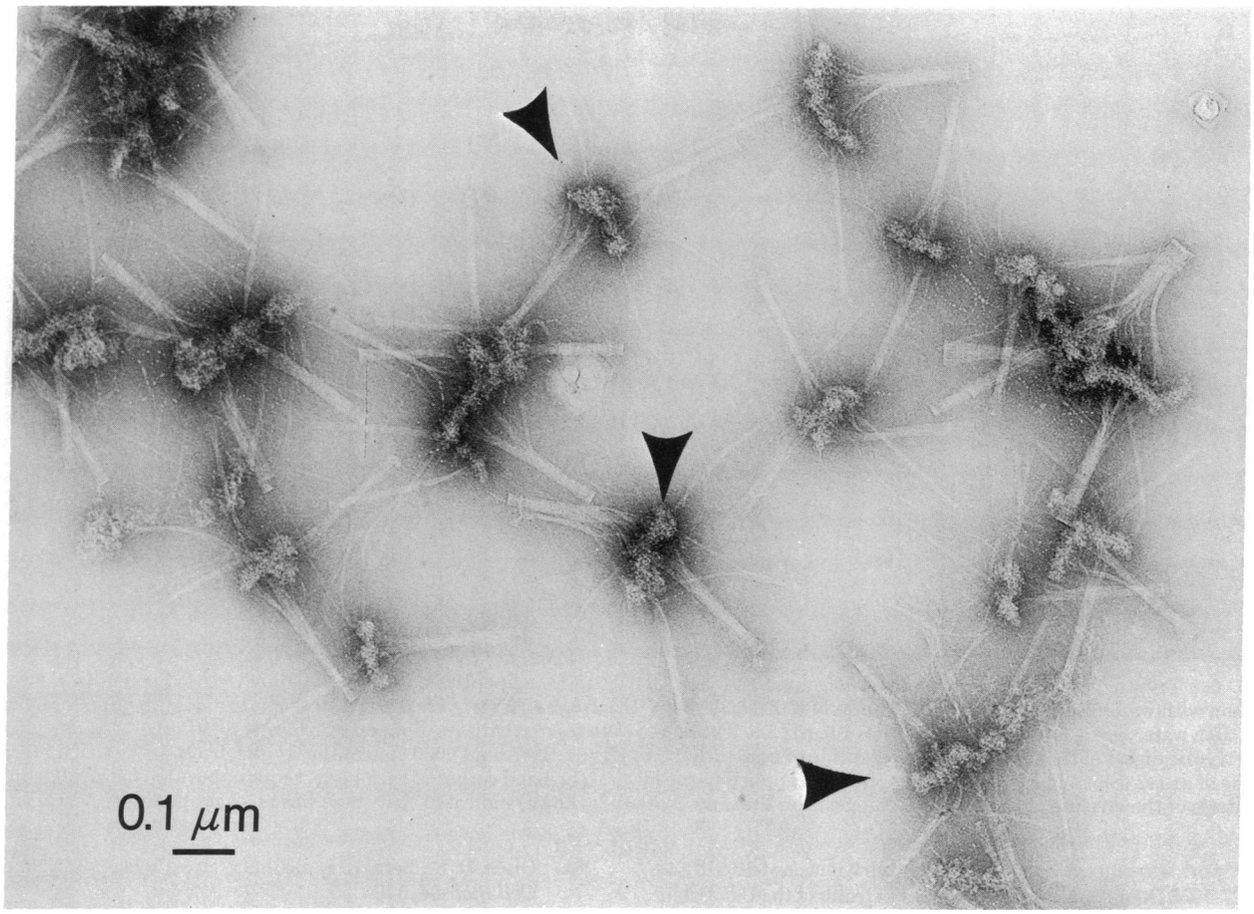
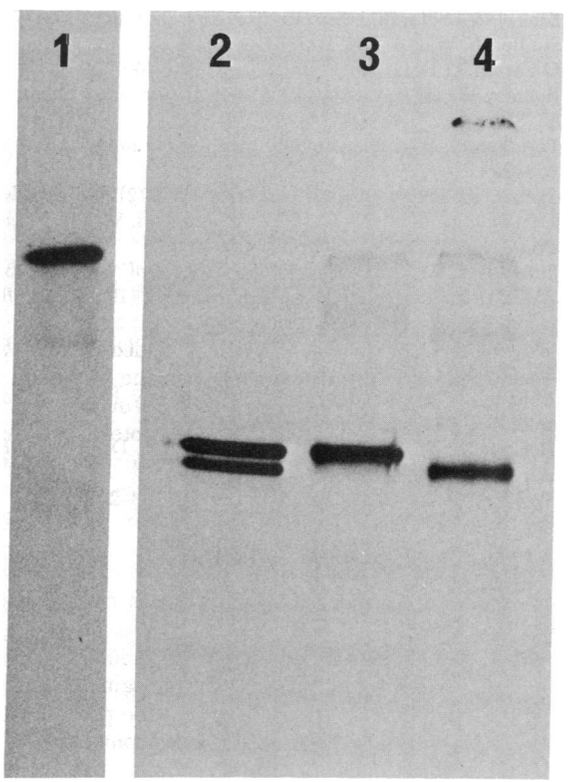


FIG. 4. SLS aggregates of procollagen from the medium of tendon cells. Procollagen was prepared with same technique as in Fig. 3 except protease inhibitors were not added before precipitation with ammonium sulfate. Also, the ammonium sulfate precipitation was repeated after the first precipitate was dissolved in 0.4 M NaCl and 0.1 M Tris buffer, pH 7.8. The samples were negatively stained with 1% ammonium molybdate. Examination of the SLS aggregates at high magnification (not shown) indicated that the NH_2-terminal extensions were missing.



chick bone procollagen but they were unable to resolve the question of whether the fragment as isolated by them contained identical or nonidentical peptides. As indicated here, reduction of the bacterial-collagenase-resistant fragment gave rise to two kinds of peptides which separate by $NaDodSO_4$ gel electrophoresi (17), and fingerprinting of the peptides demonstrated that they had different amino acid sequences. Also, based on incorporation of ^{14}C-labeled amino acids, the two peptides appeared to be present in a 2:1 ratio. It seems likely, therefore, that one of the peptides originates from the COOH-terminal end of the proα1 chain and the other from the COOH-terminal end of the proα2 chain. The function of the peptide extensions in procollagen is not defined. As suggested previously (see refs. 1, 2, and 27), however, the extensions probably direct association of the chains, and structural differences between proα1 and proα2 extensions may explain why each Type I collagen molecule contains two α1 chains and one α2 chain.

FIG. 5. $NaDodSO_4$ gel electrophoresis of COOH-terminal peptides from isolated procollagen. Slot 1: about 8700 cpm of ^{14}C-labeled trimer obtained after digestion of procollagen with bacterial collagenase. The sample was not reduced prior to application to the gel. Slot 2: about 7500 cpm of the same fragment after reduction with 2-mercaptoethanol. Slot 3: about 8000 cpm of ^{14}C-labeled peptide, which was extracted from the upper band in Slot 2 and re-examined by $NaDodSO_4$ gel electrophoresis. Slot 4: about 7000 cpm of the lower band in Slot 2. Fluorograms were prepared as described in *text*.

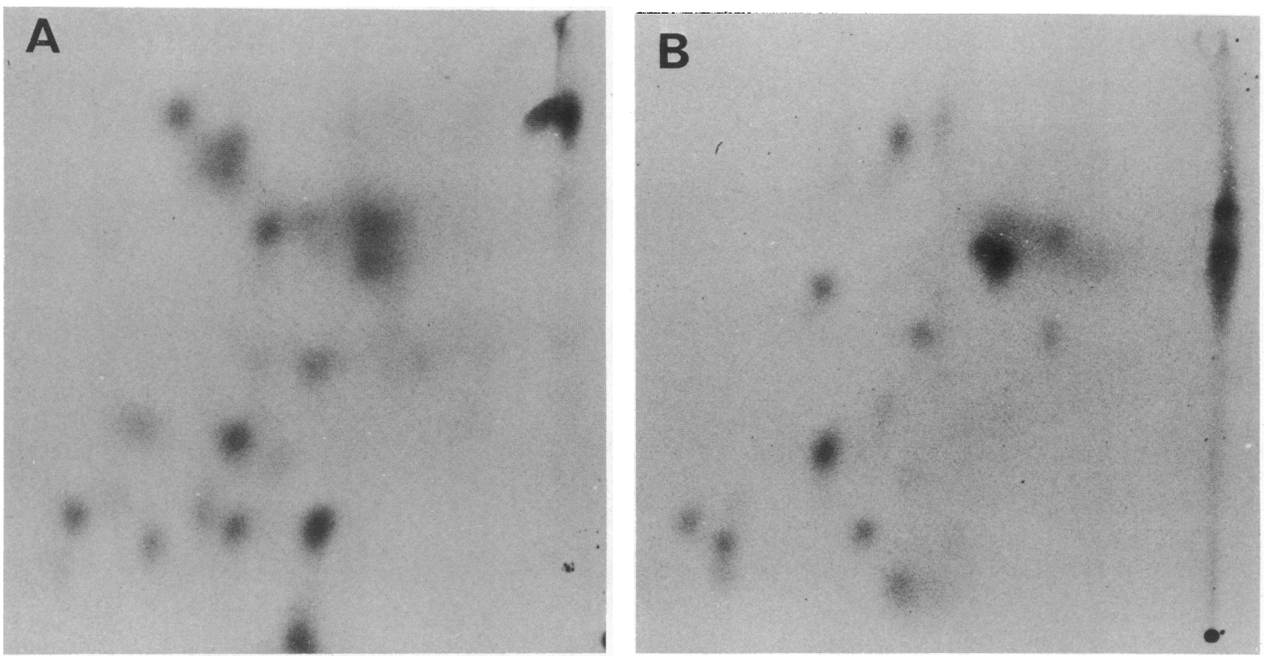


FIG. 6. Tryptic peptides obtained from collagenase-resistant COOH-terminal peptides. Chromatography was carried out in the vertical direction with water:acetic acid:pyridine:1-butanol (302:76:378:344). Electrophoresis was carried out in the horizontal direction for 60 min at 450 V with water:acetic acid:pyridine (890:100:10), pH 3.5. The plates were treated with 7% diphenyloxazole in ether (28). (A) About 8000 cpm of the tryptic digest of the COOH-terminal peptide of proα1 (Slot 3 in Fig. 5). (B) About 8000 cpm of the tryptic digest of the COOH-terminal peptide of proα2 (Slot 4 in Fig. 5). The samples were applied to the lower right-hand corner of each plate. Superimposition of the plates as well as analysis of the mixture of the two tryptic digests on the same plate (not shown) indicated that there was no overlap in the fingerprints.

This work was supported in part by Research Grants AM-16,516 and GRS-27-1938 from the National Institutes of Health of the U.S. Public Health Service. The authors gratefully acknowledge the expert technical assistance of Ms. Chris Condit.

1. Prockop, D. J., Berg, R. A., Kivirikko, K. I. & Uitto, J. (1976) in *Biochemistry of Collagen*, eds. Ramachandran, G. N. & Reddi, A. H. (Plenum Publishing Corp., New York), in press.
2. Bornstein, P. (1974) *Annu. Rev. Biochem.* **43**, 567–603.
3. Martin, G. R., Byers, P. H. & Piez, K. A. (1975) *Adv. Enzymol.* **42**, 167–191.
4. Veis, A. & Brownell, A. G. (1975) *Crit. Rev. Biochem.* **2**, 417–453.
5. Dehm, P., Jimenez, S. A., Olsen, B. R. & Prockop, D. J. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 60–64.
6. Stark, M., Lenaers, A., Lapière, C. M. & Kühn, K. (1971) *FEBS Let.* **18**, 225.
7. Goldberg, B. (1974) *Cell* **1**, 185–192.
8. Tanzer, M. L., Church, R. L., Yeager, J. A., Wampler, D. E. & Park, E.-D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3009–3013.
9. Fessler, L. I., Morris, N. P. & Fessler, J. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4905–4909.
10. Byers, P. H., Click, E. M., Harper, E. & Bornstein, P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3009–3013.
11. Olsen, B. R., Hoffmann, H.-P. & Prockop, D. J. (1976) *Arch. Biochem. Biophys.*, **175**, 341–350.
12. Dehm, P., Olsen, B. R. & Prockop, D. J. (1974) *Eur. J. Biochem.* **46**, 107–116.
13. Olsen, B. R., Berg, R. A., Kishida, Y. & Prockop, D. J. (1975) *J. Cell Biol.* **64**, 340–355.
14. Monson, J. M. & Bornstein, P. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3521–3525.
15. Fessler, L. I. & Fessler, J. H. (1974) *J. Biol. Chem.* **249**, 7637–7646.
16. Smith, B. D., Byers, P. H. & Martin, G. R. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3260–3262.
17. King, J. & Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465–477.
18. Studier, F. W. (1973) *J. Mol. Biol.* **79**, 237–248.
19. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
20. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
21. Peterkofsky, B. & Diegelmann, R. F. (1971) *Biochemistry* **10**, 988–994.
22. Weber, K., Pringle, J. R. & Osborn, M. (1972) in *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. 26, pp. 3–27.
23. Stoltzfus, C. M. & Reuckert, R. (1972) *J. Virol.* **10**, 347–355.
24. Bates, D. L., Perham, R. N. & Coggins, J. R. (1975) *Anal. Biochem.* **68**, 175–184.
25. Schofield, J. D. & Prockop, D. J. (1973) *Clin. Orthop. Rel. Res.* **97**, 175–195.
26. Murphy, W. H., von der Mark, K., McEneany, L. S. G. & Bornstein, P. (1975) *Biochemistry* **14**, 3243–3250.
27. Becker, U., Timpl, R., Helle, O. & Prockop, D. J. (1976) *Biochemistry*, **15**, 2853–2862.
28. Randerath, K. (1970) *Anal. Biochem.* **34**, 188–205.