Amino and carboxyl procollagen peptidases cleaved chick procollagen I to yield the intact amino propeptides and the disulfide-linked carboxyl propeptide, which were identified and partly characterized. The same products were released by these enzymes from the two separated fragments of procollagen produced by vertebrate collagenase, and this supports the concept that cleavage of amino and carboxyl propeptides can occur independently of each other.

The mechanism of carboxyl propeptide cleavage by enzyme preparations acting on isolated substrates was found to be closely similar to that observed in whole tissues. Specifically, while carboxyl procollagen peptidase made the initial cut in normally hydroxylated pC-collagen at random, for the subsequent two cuts the pCa1 chains were cleaved more slowly than the pCa2 chain. Intermediates and final products of carboxyl cleavage were isolated from the in vitro and in vivo systems. Comparison indicated that neither the collagen chains nor the propeptides underwent secondary, subsequent proteolytic cuts.

Partly renatured underhydroxylated procollagen was separated chromatographically into two fractions: one behaved as denatured and the other as native material by sedimentation criteria. While carboxyl procollagen peptidase correctly cleaved both the native and the denatured materials, amino procollagen peptidase only cut the native substrate; it ceased to act at temperatures at which the collagen helix became unfolded.

The release of propeptide-containing regions from procollagen by bacterial collagenase was compared with the cleavage by procollagen peptidases. The region within the amino propeptides which is a collagen helix was degraded only slowly by bacterial collagenase while in a native configuration, but was rapidly cleaved after denaturation. A tentative model is proposed in which the terminal Col 1 portion of the amino propeptide is folded back over the collagen stem.

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Nicholas P. Morris, Liselotte I. Fessler, and John H. Fessler
From the Molecular Biology Institute and Biology Department, University of California at Los Angeles, Los Angeles, California 90024

Amino and carboxyl procollagen peptidases cleaved

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Procollagen type I has amino and carboxyl propeptides (1-4). These are cleaved off sequentially yielding first an inter

mediate precursor, pC-collagen, that has lost the amino propeptides (5, 6). The next cleavage gives collagen and the disulfide-linked carboxyl propeptide, which also has been isolated after incubation of whole calvaria (3, 7) or tendon cells (8) and cell cultures (9). The disulfide-linked carboxyl propeptide dissociates on reduction into two distinct peptides pC1 and pC2 (8-11). Previously, these peptides have been inferred to be the carboxyl propeptides of, respectively, the proa1 and proa2 chains on the basis of their relative preponderance, and here we incidentally prove an unambiguous assignment of the two propeptides.

Enzymes which cleave procollagen have been shown to exist in tissue culture systems (12-14) and a neutral protease which cleaves the carboxyl propeptide has been characterized (15).

An active extract has been prepared from chick calvaria (16) and calf tissues (17) and a neutral protease which excises the amino propeptide, has been purified from calf tendon (18) and embryonic chick tendon (19). The enzyme isolated from calf tendon (18), which carries out the specific cleavage of dermatosporactic collagen, yielding collagen and the amino propeptides, has properties consistent with an enzyme which would yield an NH2-terminal pyroglutamic residue on the collagen (20).

In a preceding paper (21), we described the separation of the amino and carboxyl procollagen peptidases isolated from the medium of tendon fibroblast cultures. These enzymes process procollagen to collagen and to propeptides. In this report, we characterize the propeptides which are released from procollagen, and from underhydroxylated procollagen which is denatured at temperatures above 25°C (22).

Dermatosporactic collagen of calf and sheep has been cut previously in various ways to yield amino propeptides which have been extensively analyzed (23-25) and we compare our findings with these data (see Fig. 1). The amino propeptide of the a1 chain pN1 has a compact NH2-terminal portion which is internally disulfide-linked. The amino propeptide pN2 linked to the a2 chain lacks this region. A short stretch of collagen helix connects the compact portion to a nonhelical region which contains the proline-glutamine bond that is cleaved in the physiological formation of the amino end of collagen. The short sequence from this terminus to the start of the main collagen helix is called telopeptide (26).

A short propeptide refers to a collagen molecule which has both amino and carboxyl propeptides; the individual chains of procollagen I are: proa1 and proa2. pC-collagen refers to collagen which has carboxyl propeptides (pCa1 and pCa2). The carboxyl propeptide of proa1 is pCa1 and of proa2, pCa2. The amino propeptide of proa1 is pN1 and of proa2, pN2. When procollagen or collagen is cleaved with vertebrate collagenase, the fragments carrying the NH2 termini are the VCa pro or VCa a chains, respectively, and the fragments with the COOH termini are the VCa pro or VCa a chains, respectively.
piece of collagen helix has also been studied in the amino propeptide of bovine collagen type III (27) and appears to be exceptionally stable. Here we report on the action of the procollagen peptidases which excise the intact propeptides. We also report on the action of bacterial collagenase on the stretch of collagen helix within the amino propeptide of native and denatured, normal and underhydroxylated, chick procollagen type I and conclude that adjacent regions of the amino propeptide appear to mutually stabilize each other against proteolysis.

Collagen fiber formation in vivo could involve some interaction between propeptides of successive elements of a growing fiber, followed by excision of the propeptides. This idea raised the possibility that the cleavage of the carboxyl propeptides might be influenced by the presence of amino propeptides of other procollagen molecules. To eliminate this possibility, we cut procollagen with vertebrate collagenase and separated the native fragments: VC* procollagen containing only amino propeptides and three-quarters of the collagen main helix and the attached carboxyl propeptides. We found that the procollagen peptides cleaved the whole procollagen molecules and the separated fragments made by vertebrate collagenase equally well.

From observations on the processing of procollagen I by whole calvarial bones, Davidson et al. (10) concluded that the three carboxyl propeptides of a triplet pC-collagen molecule are cleaved in succession from their corresponding α chains. They drew the apparently paradoxical conclusion that while there was no preference between pCa1 and pCa2 chains for the first of the three cuts, the subsequent two cuts were not equally likely for α1 and α2 strands. Concurrently, we have observed the same phenomenon in intact calvaria (11) and report that it also is shown when isolated procollagen peptides act on pC-collagen. Since submitting this manuscript, we found similar kinetic effects when cleaving pC-collagen with a different enzyme. These most recent results will be published separately and support the concept of configurational change accompanying removal of the carboxyl propeptides of procollagen.

**MATERIALS AND METHODS**

The procollagen peptides were isolated as described (21). The labeled substrates were prepared by incubation of whole calvaria or tendon with 50 μCi/ml of 5-[3H]proline (49 Ci/mmol), [3H]leucine (62 Ci/mmol), [3H]tryptophan (4.6 Ci/mmol), [3H]tyrosine (42 Ci/mmol) (Becton-Dickson), or 0.5 μCi/ml of [14C]proline (260 mCi/mmol) for 90 min at 37°C. The tissue was rinsed and extracted in the presence of inhibitors of proteolysis and collagen, pC-collagen, procollagen, and carboxyl propeptides were separated by DEAE-cellulose chromatography, as described (3). The pC-collagen peak was heterogeneous, containing both pC-collagen and molecules in which the disulfide-linked carboxyl propeptides were already partly cut from the collagen chains. This mixture was used as substrate for procollagen peptidase experiments. Fractions of this DEAE-peak eluting at a higher sodium chloride concentration were enriched in these partly cut molecules and were selected for determination of the structure of intermediates. Procollagen, pC-collagen, or collagen was cleaved at 20°C for several hours with vertebrate collagenase isolated from human skin, kindly given to us by Drs. J. F. Jeffrey, A. Z. Eisen and J. Barcelo, Department of Medicine, Washington University, St. Louis, MO. The products were separated by velocity sedimentation on 5 to 20% sucrose gradients containing 0.1 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100, at pH 7.6 in a Beckman SW60 rotor at 7°C for 16 h at 65,000 rpm.

Underhydroxylated procollagen was isolated from calvaria which had been treated for 15 min with 0.1 mm α,α′-dipridyl, then labeled with radioactive amino acids in the presence of 0.1 mm α,α′-dipridyl for 60 min. The bones were extracted in 0.5 M acetic acid containing 1 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100 (pH 8.3 at 22°C). The NaCl concentration then was reduced by dialysis against the same buffer without NaCl and the mixture was chromatographed on DEAE-cellulose, as described (3). The fractions obtained were dialyzed against 0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Triton X-100 at pH 7.6. They were characterized by electrophoresis under reducing and nonreducing conditions. To distinguish whether the molecules were folded into native molecules or were present as denatured molecules, they were layered on 5 to 20% sucrose gradients containing 0.1 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100, pH 7.5, and sedimented at 50,000 rpm for 19 h at 7°C in an SW60 Beckman rotor.

The substrates were incubated in buffer at pH 7.6, containing 0.1 M NaCl, 0.05 M Tris-HCl, 0.005 M CaCl2, 0.1% Triton X-100, either with the mixed or separated procollagen peptidases (21) or with bacterial collagenase (Worthington), which had been purified (28, 29) and shown to be free of nonspecific proteases. The reactions were stopped by addition of 0.2% NaDodSO4, 0.5 μm urea, with or without dithiothreitol (10 mM), and heated at 100°C for 1 min. The products were either chromatographed as fluorograms (31), quantitated by densitometry using a Joyce-Loebl densitometer (32), or the electrophoretic migration is expressed as Rf values. For two-dimensional NaDodSO4-polyacrylamide gel electrophoresis, the first migration was performed without reduction of dithiothreitol, bands of radioactive material were detected by fluorography and cut out of the dried gel. The sections of gel were placed in a few drops of hot buffer (0.005 M Tris-HCl, 0.5 μm urea, 0.2% NaDodSO4, pH 6.8) containing 10 to 20 mM dithiothreitol (Sigma) for 30 to 60 min. Application of excess buffer was avoided to prevent loss of radioactivity by diffusion out of the gel particles. The swollen gel pieces were then placed in the wells of the stacking gel and electrophoresed in the second dimension. After the sample entered the second gel, the piece of gel and paper were usually removed.

The various forms of residual denatured chains can be separated and identified electrophoretically.

RESULTS

Separation and Identification of the Native Fragments Produced by the Action of Vertebrate Collagenase on Procollagen—In order to study the action of procollagen peptides on substrates which contained only the amino or the carboxyl portions of procollagen, we cleaved procollagen and pC-collagen with vertebrate collagenase and separated the resulting native VCA and VC\textsuperscript{B} fragments.\textsuperscript{1} This section shows that separation was achieved by velocity sedimentation, and reports on some of the properties of the fragments that allow comparison with investigations of other collagen propeptides. The action of the specific propeptides on the separated fragments has been reported, in part, previously (21).

In Fig. 2 is shown the sedimentation diagram of \([\text{\textsuperscript{3}H}]\)proline-labeled procollagen which has been cleaved with vertebrate collagenase. Since most of the proline is distributed relatively evenly throughout the collagen helix, we expect the VCA fragment to account for about three-quarters of the initial radioactivity and the VC\textsuperscript{B} fragment for approximately one-quarter. On this basis, the slower sedimenting peak is ascribed to VCA procollagen and the faster sedimenting peak to VC\textsuperscript{B} procollagen. Electrophoretic analysis of sequential sedimentation fractions, shown in Fig. 3, corroborates this conclusion. The hydrodynamic retardation caused by the highly elongated collagen helix is indicated by the finding that native procollagen sediments more slowly than VCA procollagen, which is the \([\text{\textsuperscript{3}H}]\)proline radioactivity and the VCB fragment for approximately one-quarter. On this basis, the slower sedimenting peak is ascribed to VCA procollagen and the faster sedimenting peak to VC\textsuperscript{B} procollagen. Electrophoretic analysis of sequential sedimentation fractions, shown in Fig. 3, corroborates this conclusion. The hydrodynamic retardation caused by the highly elongated collagen helix is indicated by the finding that native procollagen sediments more slowly than VCA procollagen, which is the only fragment of the initial material (Fig. 2). Similarly, the compact, native, disulfide-linked carboxyl propeptide cleaved from VC\textsuperscript{B} procollagen by carboxyl procollagen peptidase sediments still faster (Fig. 2).

The distribution of radioactivity between VCA and VC\textsuperscript{B} procollagen fragments after incorporation of several amino acids which label the propeptides is shown in Table I. Tyrosine, a carboxyl propeptide, and Trp is predominantly in the carboxyl propeptide (1-3). Tyrosine is found in the collagen telopeptides (26) as well as in the propeptides, mostly the carboxyl propeptide. Leucine is distributed equally between the collagen helix and the propeptides. Consistent with this are the NaDodSO\textsubscript{4}-polyacrylamide gel electrophoretic analyses, after reduction, of the separated VCA and VC\textsuperscript{B} fragments of procollagens labeled with \([\text{\textsuperscript{3}H}]\)leucine or \([\text{\textsuperscript{3}H}]\)tryptophan (Fig. 3). Thus, while leucine was found in all possible products generated from pro\textsuperscript{a1} and pro\textsuperscript{a2} (Fig. 3a), tryptophan occurred predominantly in the carboxyl propeptides of VC\textsuperscript{B} pro\textsuperscript{a1} and VC\textsuperscript{B} pro\textsuperscript{a2}. A small amount of tryptophan was found in the amino propeptide of VCA pro\textsuperscript{a1}, but it was absent from the amino propeptide of pro\textsuperscript{a2}, as shown by the lack of a VCA pro\textsuperscript{a2} band in Fig. 3b as compared with Fig. 3a.

Characterization of the Propeptides Cleaved from Procollagen by Procollagen Peptidases—Fig. 4 shows the propeptides released from procollagen and from the vertebrate collagenase fragments. The carboxyl propeptide is cleaved off as the disulfide-linked trimer (Channel 2), which dissociates upon reduction (Channels 3 and 4) into the propeptides identified as pC1 and pC2, which were linked to the \(\alpha_1\) and \(\alpha_2\) chains, respectively. These propeptides are readily labeled with tryptophan, leucine, cystine, and tyrosine. The electrophoretic migration of the nonreduced and reduced propeptide released by the procollagen peptidases are identical to those isolated from labeled calvaria and their incubation medium (Table II) and to pC1 and pC2 liberated by reduction from partially cut pC-collagen isolated from calvaria (Fig. 5). These carboxyl propeptides, liberated by procollagen peptidase, are slightly smaller than the propeptides attached to the telopeptides produced by the action of bacterial collagenase (Table II). The approximate molecular weights assigned to these propeptides are pC1 = 33,000, pC2 = 30,000 based on calibrations with albumin, catalase, ovalbumin, carboxic anhydrase, and chymotrypsinogen as standards.

![Fig. 2. Calvaria were labeled with \([\text{\textsuperscript{3}H}]\)proline for 50 min and the procollagen was isolated, cleaved with vertebrate collagenase at 22°C for 16 h, and the fragments were separated in an SW60 Beckman rotor at 56,000 rpm for 18 h at 7°C. The radioactivity in each fraction is plotted. The arrows indicate the position of procollagen and the carboxyl propeptide isolated from calvaria which were sedimented in a companion tube.](http://www.jbc.org/)

![Fig. 3. Electrophoretogram of the VCA and VC\textsuperscript{B} procollagen fractionated as in Fig. 2. Numbers refer to sedimentation fractions. a, procollagen was labeled with \([\text{\textsuperscript{3}H}]\)leucine. b, procollagen was labeled with \([\text{\textsuperscript{3}H}]\)tryptophan. c, collagen was labeled with \([\text{\textsuperscript{3}H}]\)proline. These were cleaved with vertebrate collagenase at 20°C for 16 h, and the fragments were separated by velocity sedimentation as in Fig. 2, and an aliquot was denatured, reduced, and electrophoresed on a NaDodSO\textsubscript{4}-7% acrylamide slab gel. The fluorogram is shown.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Labeled amino acid distribution in the two fragments produced by vertebrate collagenase cleavage of procollagen</th>
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<tbody>
<tr>
<td>Procollagen labeled with (\text{[\textsuperscript{3}H}])proline</td>
<td>VC\textsuperscript{B} procollagen</td>
</tr>
<tr>
<td>(\text{[\textsuperscript{3}H}])Proline</td>
<td>6,000</td>
</tr>
<tr>
<td>(\text{[\textsuperscript{3}H}])Tryptophan</td>
<td>8,250</td>
</tr>
<tr>
<td>(\text{[\textsuperscript{3}H}])Tyrosine</td>
<td>13,300</td>
</tr>
<tr>
<td>(\text{[\textsuperscript{3}H}])Leucine</td>
<td>6,700</td>
</tr>
</tbody>
</table>
amino acids were found in pN1, the amino propeptide of the peptides (not shown). While the electrophoretic mobility of we used, separately, \(^{[7}H\)leucine-labeled VCA procollagen and chains. In contrast, the VCB procu substrate did not give these VCB procollagen, the fragments into which procollagen is cut by the action of procollagen peptidases. As substrates \[^{[3}H\]leucine, but not by cystine or tryptophan, while all three were made to label the amino propeptides. The amino propeptide portions of, respectively, the cul and (~2 collagen by vertebrate collagenase. Channels 5 and 6 of Fig. 4 show their resolution at this polyacrylamide concentration. Channel C, electrophoresis of reduced carboxyl propeptides. The complex of the three mutually disulfide-linked carboxyl propeptides [(pC1)2pC2] migrated only a short distance, and uncleaved substrate forms the top band, which just entered the gel. Channel 3, electrophoresis of reduced carboxyl propeptides. The starting material was the middle band of a duplicate electrophoretogram run in the same manner as Channel 2 (as indicated by arrows). The internally disulfide-linked pN1 propeptide migrated furthest through the gel. The complex of the three mutually disulfide-linked carboxyl propeptides [pC1]pC2) migrated only a short distance, and uncleaved substrate forms the top band, which just entered the gel. Channel 3, electrophoresis of reduced carboxyl propeptides. The sample was reduced before electrophoresis. Partial cleavage of the VC4 procollagen left some VC4 proa1 and VC4 proa2 chains which are seen as two upper bands. Channel 5, amino propeptides pN1 and pN2 produced when procollagen peptides acted on isolated \(^{[7}H\)leucine-labeled VC4 procollagen for 12 h at 20°C. The sample was reduced before electrophoresis. Arrow indicates faint, but reproducibly distinct pN2 peptide. In the upper portion of the electrophoretogram are uncleaved substrate VC4 proa1 and VC4 proa2 chains (labeled VC4 proa) and the VC4 a1 and VC4 a2 chains produced from them by procollagen peptidase. The closely similar electrophoretic mobilities of VC4 proa2 and VC4 a1 precluded their resolution at this polyacrylamide concentration. Channel 6, same material as Channel 5 but electrophoresed without reduction. Incorporation studies with several radioactive amino acids were made to label the amino propeptides. The amino propeptide pN2 (cleaved from the a2 chain) was labeled by \(^{[7}H\)leucine, but not by cystine or tryptophan, while all three amino acids were found in pN1, the amino propeptide of the a1 chain. Fig. 4 illustrates the release of these amino propeptides by the action of procollagen peptidases. As substrates we used, separately, \(^{[7}H\)leucine-labeled VC4 procollagen and VC4 procollagen, the fragments into which procollagen is cut by vertebrate collagenase. Channels 5 and 6 of Fig. 4 show that VC4 procollagen, the fragment which contains the amino end of procollagen, was cleaved by amino procollagen peptidase into the \(^{[7}H\)leucine-labeled amino propeptides pN1 and pN2, and also gave the corresponding VC4 a1 and VC4 a2 propeptide portions of, respectively, the a1 and a2 collagen chains. In contrast, the VC4 proa substrate did not give these peptides (not shown). While the electrophoretic mobility of pN2 was not changed by reduction with dithiothreitol, the amino propeptide pN1 moved more slowly after reduction (Channels 5 and 6 of Fig. 4). This behavior was also shown by \(^{[7}H\)tryptophan-labeled pN1 which had been cleaved from whole procollagen by procollagen peptidases (Channels 1 and 2 of Fig. 4). As cystine also labels pN1 (not shown), we conclude that disulfide bridges hold this peptide in a conformation which gives it greater mobility in this electrophoresis system before, as compared with after, reduction. Any attempt at assignment of molecular weights of amino propeptides on the basis of electrophoretic mobility is further complicated by their content of collagen sequences. The greater mobility of pN2 than pN1, lower incorporation of \(^{[7}H\)leucine and absence of tryptophan and cystine are all consistent with pN2 being a smaller peptide than pN1.

Treatment of the VC4 procollagen with the amino procollagen peptidases and the VC4 procollagen with carboxyl procollagen peptidase yields propeptides with the same electrophoretic mobility as those obtained with the mixed enzymes. When procollagen is treated with procollagen peptidases first and thereafter bacterial collagenase is added and incubation is continued at 35°C for 4 h, the carboxyl propeptide remains unchanged. The pN1 propeptide is reduced in size to pN2, which is degraded by bacterial collagenase. pN2 is degraded by bacterial collagenase into noncollagenous peptide, called pN1-Co1 1, based on the characterization of this peptide isolated from dermatosporac collagen (23). pN2 is degraded by bacterial collagenase into small peptides.

Characterization of pC-collagen and Partially Cut pC-collagen—The reasons for the following detailed analyses are 1) to show the relationship of the cleavage products obtained with the enzyme preparation to those which arise in whole tissue, and 2) to prove the assignment of the carboxyl propeptides to the two a chains.

**Table II**

<table>
<thead>
<tr>
<th>Enzymatic cleavage products from procollagen</th>
<th>Intact calvaria</th>
<th>Bacterial collagenase</th>
</tr>
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<tbody>
<tr>
<td>Carboxyl propeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonreduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pC1)pC2</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reduced</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>Amino propeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonreduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN1</td>
<td>0.72</td>
<td>0.68</td>
</tr>
<tr>
<td>pN1-Col 1</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>pN2</td>
<td>0.92</td>
<td>0.90</td>
</tr>
<tr>
<td>Reduced</td>
<td>0.90</td>
<td></td>
</tr>
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</table>

*The carboxyl propeptides present in the medium and those isolated from the whole calvaria were indistinguishable.
+ The identical findings were obtained with VC4 procollagen and with procollagen.
+ These values are the center of rather broad bands, suggesting some heterogeneity in the size of these peptides.

**Fig. 4.** Electrophoretic analyses of propeptides produced by the action of procollagen peptidases on whole procollagen (Channels 1, 2, 3) and on the separated VC4 and VC5 fragments of procollagen produced by prior action of vertebrate collagenase (Channels 4, 5, 6). As indicated, some materials were reduced with 10 mM dithiothreitol before electrophoresis in the 12% polyacrylamide slab containing 0.2% NaDodSO4 and 0.5 M urea. Aliquots of some disulfide-linked materials were first isolated from duplicate electrophoretograms and, after reduction, served as starting materials for this experiment. Channel 1, electrophoresis of reduced pN1 peptide. This material was obtained from an electrophoretic separation run in the identical manner as Channel 2 (as indicated by arrows): the band labeled pN1 in Channel 2 was cut out, reduced, and re-electrophoresed. Note the unusual effect of reduction causing a decrease of the electrophoretic mobility of pN1. Channel 2, procollagen labeled with \(^{[7}H\)tryptophan was incubated with procollagen peptides at 20°C for 12 h and the products were electrophoresed. The internally disulfide-linked pN1 propeptide migrated furthest through the gel. The complex of the three mutually disulfide-linked carboxyl propeptides [pC1]pC2) migrated only a short distance, and uncleaved substrate forms the top band, which just entered the gel. Channel 3, electrophoresis of reduced carboxyl propeptides. The starting material was the middle band of a duplicate electrophoretogram run in the same way as Channel 2 (as indicated by arrows). Channel 4, carboxyl propeptides pC1 and pC2 produced when procollagen peptides acted at 20°C for 8 h on isolated, \(^{[7}H\)lleucine-labeled VCA procollagen for 12 h. Channel 4, carboxyl propeptides pC1 and pC2 produced when procollagen peptides acted at 20°C for 8 h on isolated, \(^{[7}H\)lleucine-labeled VCA procollagen for 12 h. Channel 4, carboxyl propeptides pC1 and pC2 produced when procollagen peptides acted at 20°C for 8 h on isolated, \(^{[7}H\)lleucine-labeled VCA procollagen for 12 h.
The pC-collagen fraction was isolated by DEAE-chromatography from calvaria which had been incubated for 60 min with radioactive amino acids, and so was the carboxyl propeptide from the incubation medium. Reduction released pC1 and pC2 propeptides from the isolated, partially cleaved pC-collagen, and Fig. 5 shows that these propeptides have the same electrophoretic mobilities in 12.5% polyacrylamide slab gels as those isolated from the medium. However, pC-collagen sometimes has a weak unidentified band pC' which moves slightly slower than pC1. This band was never observed in electrophoretograms of the fully cleaved carboxyl propeptides recovered from the medium.

The following sequence of experiments proved assignment of individual electrophoretic bands to the propeptides pC1 and pC2, and to the intermediates produced from pC-collagen by successive action of carboxyl procollagen peptidase. Electrophoretic analysis, without reduction, of the denatured pC-collagen fraction gave the bands shown in Fig. 6A. From parallel channels, the bands were cut out, reduced, and electrophoresed on either a 7.5% polyacrylamide slab gel or a 12.5% polyacrylamide slab gel to separate the propeptides pC1 and pC2. The results, which are summarized, permit the identification of each band as shown in the diagram, Fig. 6B. Band a gives pCα1 and pCα2 in the proportions present in the trimer [(pCα1)pCα2]. Band b shows only pCα1 and pC2 in the second dimension, and so must lack pα2, hence the structure is [(pCα1)pC2]. Band c contains both pCα1 and pCα2 in approximately equal amounts and pC1, so must have lost dissociation one pα1 chain to account for its migrating more rapidly than the trimer. The structure is [(pCα1)(pCα2)−(pC2)]. Band d contains only pCα1 and pC1 and pC2 and therefore must have lost both an pα1 and an pα2 chain upon denaturation. The structure is [(pCα1)(pC1)(pC2)]. Finally, Band e has lost both α1 chains and contains only pCα2 and pC1, yielding a structure of [(pCα2)(pC1)]. Bands f and g coelectrophorase with α1 and α2 and in the second dimension identified α1 and α2 are shown. From a second nonreduced gel, Bands a to g were cut out, reduced, and electrophoresed on a 12.5% gel to resolve the pC1 and pC2 bands clearly and the results are tabulated in B. These data are the basis for the identifications given.

Cleavage of pC-collagen by Carboxyl Procollagen Peptidase—pC-collagen containing partially cleaved intermediates served as substrate for procollagen peptidase. After increasing times of incubation, the products were electrophoresed on a 4% polyacrylamide gel. With progressive reaction time, the relative intensities of the bands changed, but all bands retained the RF values observed in the original, partially cleaved substrates, Table III. This indicates that the enzyme produces
in vitro the same intermediates present in vivo. Fig. 7 shows the change with time in the relative amount of each inter-
mediate. The intermediate forms were already present at zero
in vitro the same intermediates present in vivo.

Each intermediate product of the action of carboxyl procolla-
genase on p-C collagen is dissociated due to the action of
NaDodSO₄ into a disulfide-linked complex of pCa chains and
carboxyl propeptides, and, separately, α chains which had
previously been associated with the triple helical complex by
noncovalent linkage. As most of the radioactivity of the
[³H]proline-labeled substrate resides in the collagen portion of
a pCa chain, measurement of the relative radioactivity of
electrophoretic bands b and c of Fig. 6A by densitometry of
fluorograms only gives two-thirds of the radioactivity which
was present in the cleavage intermediate before detergent
and electrophoresis (see also Fig. 6B). The remaining
one-third of the original radioactivity is found in α chains
which contributed to Bands f and g of Fig. 6A. In order to
calculate the relative amounts of cleavage intermediates in a
given sample, as plotted in Fig. 7, the densitometric peak
areas of Bands b and c therefore had to be corrected. To
obtain the radioactivity of the corresponding triple helical
molecules would have required multiplication of the densito-
metric peak areas of Bands b and c by the factor 3/2. In order
to minimize cumulative errors of calculation, we instead chose
to use as a measure of each native triple helical species one-
third of its total radioactivity. Thus, the content of initial,
uncut p-collagen [(pCa1)(pCα2)(pCα)] was expressed as one-third
of the densitometric peak area of Band a, and the
intermediate [(pCa1)(pCα2)(pCα)] as half the peak area of
Band b, and the intermediate [(pCa1)(αCpCα)(pCα)] as
half the peak area of Band c. Reference to Fig. 6B shows that,
correspondingly, the peak area of Band d was taken
without correction as a measure of the intermediate
[(pCa1)(αCpCα)(α2CpC2)] and peak area of Band e as a
measure of [(α1CpC1)(pCα)(pCα2)].

The relative quantity of finished, triple helical collagen
molecules present in an incubation mixture before
denaturation by NaDodSO₄, and electrophoresis was taken as one-third
of the summed areas of Peaks f and g, after a correction. The
correction was to subtract the radioactive contributions of α1 and
α2 chains which had been associated with the complexes
denoted by b, c, d, and e of Fig. 6B in the native form, but
which upon electrophoresis migrated to Bands f and g. Indi-
vidual contributions to Bands f or g were calculated according
to the structures shown in Fig. 6B. For example, as Band b
represents [(pCa1)(pCα2)], it arose from the native cleavage
intermediate [(pCa1)(α2CpC2)] and, after electrophoresis, the
α2 chain set free on denaturation contributed half as much
radioactivity to Band g as was found by densitometric
measurement of Band b. For these calculations, it was assumed
that α1 and α2 chains had equal proline content and that by
comparison the proline content of the carboxyl propeptides
was negligible.

In general, intermediates lacking one α chain, Curves b and
c, accumulate to the greatest extent at 30 min. Of the inter-
mediates lacking two α chains, [(pCa1)(pCα1pCα2)],
Curves e, d, increases to about 60 min, while [(pCα2)(pCα1)].
Curves c, d, is found at very low levels and does not change much with time.
The ratio of the amounts of each of the homologous pairs of
intermediates at each time point is shown in Table IV.
[(pCa1)(pCα1)(pCα2)]. Curves d, increases with time relative to
[(pCα2)(pCα1)], Curves c. The ratio of c/b, that is the amount
of [(pCa1)(pCα2)(pCα1)] relative to [(pCa1)(pCα2)], is
approximately 2 at time zero and decreases to about 1 with time.
If cleavage of each α chain were random with respect to order,
and if the rate constants for cleavage of pCa1 and pCα2 were
the same, then the relative amounts of each of the pairs of
intermediates c/b and d/e would be in a 2:1 ratio. The reason
for this discrepancy is yet unexplained.

**Products of Digestion of Procollagen by Bacterial Colla-**
TABLE IV

The ratio of the amounts of the homologous pairs of intermediates in the in vitro processing of pC-collagen as a function of the time of incubation with procollagen peptidase

The data are taken from Fig. 7 with the corrections as described in the text. The table shows the ratio of the value of Curve c to b, or d to e at each of the incubation times.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Ratio c/b</th>
<th>Ratio d/e</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.3</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>1.4</td>
<td>4.3</td>
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<tr>
<td>20</td>
<td>1.35</td>
<td>3.3</td>
</tr>
<tr>
<td>30</td>
<td>1.2</td>
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<tr>
<td>45</td>
<td>1.15</td>
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<tr>
<td>60</td>
<td>1.1</td>
<td>8.5</td>
</tr>
<tr>
<td>90</td>
<td>0.9</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Procollagen Propeptide Release

The peptides released from procollagen by mild digestion with bacterial collagenase contain both the propeptides and the telopeptides, and therefore these chains are slightly longer than the propeptides cleaved off by the procollagen peptidases, Table II. This observation was made initially by Murphy et al. (7), who compared the physiologically cleaved carboxyl propeptide with the carboxyl peptide released by bacterial collagenase, and our findings are in agreement. Similarly, the amino propeptides linked to the corresponding telopeptides are released from procollagen by mild treatment with bacterial collagenase. Thus, when [3H]-leucine-labeled procollagen or VCA procollagen are digested with bacterial collagenase for 30 min, a period long enough to cleave the main collagen molecule completely into small peptides, two amino propeptide bands are seen.

However, prolonged digestion with bacterial collagenase shows that these amino propeptides contain a collagenous sequence which is less accessible to proteinase than the main collagen helix, and by analogy with other findings (23, 33), we denote the final resistant peptide to be pN1-Col I, see Fig. 1. The time course of cleavage of native [3H]-leucine-labeled procollagen by bacterial collagenase is shown in Fig. 8. The control channel shows that collagen present in a 5- to 10-fold excess is cleaved almost completely within 30 min. At short incubation times, procollagen is converted to the carboxyl propeptides with the telopeptides and to pN2 with the telopeptide and pN1 with the telopeptide, and at longer incubation times the former two are converted to pN1-Col I and smaller peptides. When these various bands were cut out and re-electrophoresed before and after reduction the following was observed: pN2 with the telopeptide did not alter its electrophoretic migration as is shown in Table II. As would be expected, pN2 with the telopeptide is seen as a radioactive band when the enzyme acts on leucine or tyrosine-labeled procollagen, but not when tryptophan-labeled procollagen serves as substrate; whereas pN1 with the telopeptide and pN1-Col I are seen after digestion of all these labeled substrates. Heterogeneity of pN1 with the telopeptide is indicated by the width of this band at the shorter incubation times and this might be due to different sized a chain residues which might still be attached to the telopeptide. Furthermore, some minor slower migrating bands appear when procollagen is incubated with bacterial collagenase, but the nature of these is unknown.

When procollagen is warmed at 60°C for 30 min, which denatures the collagen helix of the propeptides, bacterial collagenase rapidly degrades the amino propeptides into pN1-Col I and smaller peptides, Fig. 8.

Cleavage of Underhydroxylated Procollagen—Calvaria were preincubated with a,a'-diptyridyl and then labeled with either [3H]-tryptophan or [3H]-leucine in the presence of a,a'-diptyridyl for 60 min. The extract of the bones was dialyzed extensively at 0°C to permit folding of the procollagen molecules. Then, these were chromatographed on DEAE-cellulose columns in the same manner described for hydroxylated collagens (3). Fig. 9 shows such a chromatogram. The procollagen was found to be present not as a single peak, as expected, but as two separate peaks. Both of these consist predominantly of the underhydroxylated procollagen trimer [(proa1)-(proa2)]2, as shown by electrophoresis of the reduced proa1 and proa2 chains. Previously (22), sedimentation analysis had been employed to distinguish helically folded and denatured procollagen molecules. Sedimentation analysis of Peak 1 procollagen shows that it consists of partly denatured molecules which sediment faster than 3.7 S and in Peak 2 the molecules appear to be renatured and helically folded, sedimenting as a sharp peak, 3.7 S. The latter material can be precipitated from a 0.2 M acetic acid solution by addition of 5% NaCl whereas the denatured molecules remain soluble. The renatured procollagen of Peak 2 is underhydroxylated and is denatured by warming above 25°C.

When the underhydroxylated procollagen of Peak 2 is denatured at 35°C, it serves as substrate for the carboxyl procollagen peptidase and the carboxyl propeptide released is indistinguishable from the peptide cleaved from hydroxylated procollagen (Table V). Similarly, bacterial collagenase cleaves underhydroxylated procollagen into the expected carboxyl propeptides with the telopeptide. The amino procollagen peptidase does not release the amino propeptides from this denatured underhydroxylated procollagen. The amino propeptide of the a1 chain is cleaved to pN1-Col I within 15 min of incubation with bacterial collagenase and no pN2 amino propeptide is seen. The collagen segment of this propeptide appears not to be folded at 35°C and is readily accessible to the enzyme.

![Fig. 8. Time studies of the cleavage of procollagen and collagen by bacterial collagenase. The [3H]-leucine-labeled substrates were incubated at 30°C for the indicated times with a constant amount of bacterial collagenase. The substrates were native procollagen, denatured procollagen, which had been warmed to 80°C for 15 min and then cooled to 0°C before incubation, and native collagen. At the end of the incubation, 2% NaDodSO4 and 0.5 M urea, final concentration, were added and the samples were heated 2 min at 100°C. Electrophoresis for the procollagens was on a 12.5% polyacrylamide slab gel and for collagen on a 10% polyacrylamide slab gel. The fluorogram is shown. Samples of procollagen and collagen were also incubated for the above times without bacterial collagenase in the presence of 5 mM CaCl2 (not shown) and no degradation of the material was observed.](https://www.jbc.org/)

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By guest on October 31, 2017
Procollagen Propeptide Release

Table V

Propeptides released from hydroxylated and underhydroxylated procollagen

<table>
<thead>
<tr>
<th>Procollagen peptides</th>
<th>Bacterial collagenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylated procollagen</td>
<td>Hydroxylated procollagen</td>
</tr>
<tr>
<td>Carboxyl propeptides</td>
<td>Denatured hydroxylated procollagen</td>
</tr>
<tr>
<td>pC1</td>
<td>0.50</td>
</tr>
<tr>
<td>pC2</td>
<td>0.54</td>
</tr>
<tr>
<td>Amino propeptides</td>
<td></td>
</tr>
<tr>
<td>pN1</td>
<td>0.57</td>
</tr>
<tr>
<td>pN1-Col 1</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* These bands are broad and the $R_f$ is an average value.

DISCUSSION

The carboxyl propeptides are released by procollagen peptides as the disulfide-linked trimer (pC1).pC2. This is indistinguishable from that isolated from whole calvaria. The same pC1 and pC2 propeptides were released by reduction of pC-collagen which had been partially cleaved. The enzymes cut the same disulfide-linked trimer (pC1).pC2 from hydroxylated and underhydroxylated native or denatured procollagen. To ascertain that the enzymes cut procollagen at the correct cleavage site, it will be necessary to obtain the amino acid sequence of the NH₂ terminus of the carboxyl propeptide and of the telopeptide. Bacterial collagenase produces slightly larger material, presumably containing the telopeptides of the carboxyl ends of the collagen chains, in agreement with previous findings (7).

The intermediates produced in the sequential cleavage of the carboxyl termini of the three-stranded pC-collagen molecule are the same in whole tissue and by separate interaction with procollagen peptides, and allow unambiguous assign-

ment of the carboxyl propeptides pC1 and pC2. We have not tried to resolve the carboxyl procollagen peptides activity into separate enzymes for cutting each carboxyl propeptide. The overall course of the cleavage and the rate of making the first cut into the triplet pC-collagen molecule are consistent with a single enzyme without strand preference. The second cut is made more frequently in a pCα2 chain than would be expected by random occurrence among the remaining intact chains. Although experimental difficulties prevent a full investigation of the system, repeated measurements by David-son et al. (10) and ourselves (11) indicate this in whole tissues and hence also with the isolated system. As carboxyl procollagen peptidase can probably act both on molecules in solution and on aggregates (21), the above kinetic differences could be related to varying accessibility of propeptides to cleavage, if this proceeded during assembly of microfibrils.

As in vitro translation of chick mRNA shows that there is a signal or leader sequence to procollagen chain (34) which will be cleaved off soon after synthesis, conclusions about the amino propeptides are governed by the source of procollagen. The materials used here presumably are procollagen molecules without the signal peptide. The amino propeptides of chick procollagen appear to be analogous in structure to the amino propeptides derived from dermatosparatic collagen (23-25), see Fig. 1. The data presented here show that pN1 consists of an NH₂-terminal, compact, internally disulfide-linked pN1-Col 1 fragment, which is followed by a short segment of a collagenous peptide, and an intermediate sequence, containing the telopeptide, which is linked to the α1 chain. pN2 lacks the compact, cystine-containing portion.

The amino procollagen peptides cleave off the intact amino propeptides, pN1 and pN2 from hydroxylated, native procollagen or from the vertebrate collagen fragment, VCα procollagen. When underhydroxylated procollagen, which is denatured at 35°C, serves as substrate during a comparable incubation period, these amino propeptides are not cut off and pN-collagen accumulates.

The amino propeptides contain a segment of collagen helix which is more resistant to bacterial collagenase degradation than the main collagen helix (Fig. 1). This difference is not observed after heating to 60°C or when underhydroxylated procollagen is used at 35°C. Although this resistance could be an inherent property of this short helix other portions of the amino propeptide could also protect it against bacterial collagenase digestion.

Specifically, we consider a model in which the Col 1 portions are folded back over the collagen helix of the amino propeptide and approximately cover it. From the circular dichroism and ultracentrifuge studies of Engel and associates (27, 35, 36) the Col 1 portion of dermatosparatic sheep type I and of bovine type III pN-collagens appear as distinctly elongated structures (axial ratio 1:10), about 140 Å long, with structural elements of a distorted β conformation, as well as aperiodic elements, held by five disulfide bridges in a configuration which is remarkably resistant against denaturation. The collagen stems of these amino propeptides consist of three chains, each of about 45 amino acid residues (27) and therefore about 130 Å long.

We calculate that the volume of the approximately 100 amino acid residues of each Col 1 peptide (27, 35) is sufficient to form a layer about 5 Å thick that would extend along the length of such a collagen helix and cover one-third to one-half of its surface, and correspondingly this could be formed from a relatively extended polypeptide, largely in a β configuration, folded back on itself once or twice.

The length of amino propeptides measured by electron
microscopy of segment long spacing crystallites is in the range 130 to 200 Å (chick I procollagen, 130 Å (4, 37, 38); bovine: dermatosparactic pN-collagen I, 200 Å (39); III pN-collagen, 160 Å (40); chink pN collagen, 170 Å (41)) and dermatosparactic sheep collagen fibers contain a 180 Å extension (42). The lower limit of this range would require some folded over structure of an elongated Col 1 and the upper values are not inconsistent with a folded over form.

Kinetic studies indicate that the Col 1 portion does not influence the rate of collagen helix formation or stability of an artificial amino propeptide trimer [pN1(d)], but the naturally occurring [pN1(d) pN1] has not been studied (53). There is little interaction between isolated Col 1 fragments (53) and their configuration changes significantly on reduction (35).

There are substantial similarities between the amino propeptides of type I and III procollagens (44), and the collagen helix of chick (45) and bovine (27, 46) amino propeptide III is also resistant to bacterial collagenase, unless the propeptide helix of chick (45) and bovine (27, 46) amino propeptide III is occurring [pN1]zpN~ has not been studied (35). There is influence the rate of collagen helix formation or stability of an attic sheep collagen fibers contain a 180 Å extension (42). The dermato-}

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N P Morris, L I Fessler and J H Fessler


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