Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H. (1970), *Biochemistry 9*, 1997.
Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972a), *Biochemistry 11*, 4882.
Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972b), *Biochemistry 11*, 4892.
Hartley, B. S. (1964), *Nature (London) 201*, 1284.
Hartley, B. S., and Shotton, D. M. (1971), *Enzymes 3*, 323.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11*, 4493.
Jackson, C. M., and Hanahan, D. J. (1968), *Biochemistry 7*, 4506.
Leveson, J. E., and Esnouf, M. P. (1968), *Brit. J. Haematol. 17*, 173.
Lundblad, R. L., and Davie, E. W. (1965), *Biochemistry 4*, 113.
Magnusson, S. (1971), *Enzymes 3*, 277.
Milstone, J. H. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 742.
Robbins, K. C., Arzadon, L., Bernabe, P., and Summaria, L. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 446 Abstr.
Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10*, 2743.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225*, 811.
Walsh, K. A., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Wright, I. (1959), *J. Amer. Med. Ass. 170*, 325.

Structural Studies on Cartilage Collagen Employing Limited Cleavage and Solubilization with Pepsin†

Edward J. Miller

ABSTRACT: Insoluble cartilage collagen was prepared as the residue from the sternal cartilages of 10-week-old chickens by exhaustive extraction with 1.0 M NaCl at neutral pH and 0.5 M acetic acid. The extraction procedures were totally ineffective in solubilizing cartilage collagen but were useful as a means of removing proteoglycan components of the tissue. Amino acid analyses of the insoluble cartilage collagen residue revealed an amino acid composition closely resembling that of purified α1(II) chains. Characterization of the cyanogen bromide (CNBr) cleavage products derived from insoluble cartilage collagen indicated that they are, for the most part, qualitatively and quantitatively identical with those previously observed in CNBr digests of α1(II) prepared from soluble cartilage collagen. However, two additional CNBr peptides (designated peptides 14 and 15) comprising a total sequence of 21 amino acids derived from a nonhelical region of the cartilage collagen molecule were identified in the present study. Incubation of insoluble cartilage collagen in 0.5 M acetic acid containing pepsin (ratio of collagen: enzyme = 10:1, w/w) at 4° for 18 hr solubilized 60–70% of the collagen. Characterization of the pepsin-solubilized cartilage collagen with respect to chain composition, molecular weight of the component α chains and CNBr cleavage products of the chains indicated that the collagen was solubilized as monomeric molecules of the chain composition, $\{\alpha 1(II)\}_3$, and that the proteolytic activity of pepsin on the native cartilage collagen molecule is confined to relatively short sequences represented by the CNBr peptides, 1, 4, 14, 15, and the COOH-terminal portion of peptide 7. These results indicating that the cited sequences do not participate in collagen helix formation and that they are localized at the extremities of the α1(II) chains comprising the cartilage collagen molecule have been used, in conjunction with additional data on the location of peptides 1 and 4, to establish that the order of the CNBr peptides in the carboxy-terminal region of the α1(II) chain is: 7-14-15. These results further indicate that failure to detect peptides 14 and 15 in the CNBr cleavage products of α1(II) prepared from soluble cartilage collagen resulted from nonspecific proteolytic activity during extraction and purification of the collagen. It is proposed that the mechanism whereby the proteolytic activity of pepsin alters the solubility properties of cartilage collagen involves, at least in part, the degradation of the sequence represented by peptide 4, thus effectively eliminating a site of intermolecular cross-linking known to occur in this sequence.

In recent years, conclusive evidence has been presented indicating that cells of higher organisms possess several structural genes for collagen synthesis. Furthermore, current information indicates that expression of the genes for collagen synthesis is highly selective in certain cell types giving rise to some degree of specificity with respect to the type of collagen molecule found in various tissues. Cartilage collagen, for example, is comprised predominantly of molecules containing three identical α chains, designated α1(II) chains to distinguish them from the α1(I) and α2 chains common to the collagen in several other tissues such as bone, skin, and tendon (Miller and Matukas, 1969). Additional studies have shown that the chain composition of the collagen molecules in a variety of cartilaginous structures may be characterized as $\{\alpha 1(II)\}_3$. These include chick sternal (Trelstad *et al.*, 1970; Miller, 1971a), chick growth plate (Toole *et al.*, 1972), human

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growth plate (Miller *et al.*, 1971), and bovine articular (Strawich and Nimni, 1971) cartilages.

The present studies were prompted by recent electron microscope investigations demonstrating that treatment with pepsin cleaves an approximately ten-nm segment from the carboxy-terminal region of the native cartilage collagen molecule (Stark *et al.*, 1972). It was postulated that intermolecular cross-linking in molecules of the chain composition, $\{\alpha 1(II)\}_3$, might involve nonhelical regions and that treatment of intact cartilage slices with pepsin would provide an effective means of solubilizing a substantial proportion of the collagen. The collagen rendered soluble in this fashion has been characterized with respect to chain composition, amino acid composition, molecular weight of the denatured chains, and cyanogen bromide (CNBr) peptides derived from the chains.

In conjunction with concomitant studies on the CNBr peptides derived directly from insoluble cartilage collagen, the data provide a more detailed evaluation of the activity of proteolytic enzymes on cartilage collagen at the molecular level and serve to indicate the location of several CNBr peptides in the $\alpha 1$(II) chain. The data further indicate that the cartilage collagen molecule contains at least two regions of nonhelicity, one located in the COOH-terminal region and one in the NH_2-terminal portion of the molecule.

Materials and Methods

Preparation of Collagen. The collagen employed as the starting material in the present studies was prepared from the sternal cartilages of 10-week-old chickens. For this purpose, the tissues were carefully stripped of perichondrium and diced into small slices, and the majority of the proteoglycan components were removed from the fresh cartilage slices by daily extraction with constant stirring at 4° in approximately five volumes of 1.0 M NaCl (pH 7.5, 0.05 M Tris) for 5 successive days. Following these extraction procedures, the slices were thoroughly rinsed at 4° in distilled water and extracted with 0.5 M acetic acid employing the same protocol as outlined above for the neutral salt solvent. Subsequent to acid extraction, the slices were collected by centrifugation at 5000*g*, resuspended in 0.5 M acetic acid and lyophilized. The material thus obtained was operationally defined as insoluble cartilage collagen.

Solubilization with Pepsin. Cartilage collagen was routinely solubilized by suspending 1 g of lyophilized cartilage slices in 100 ml of 0.5 M acetic acid containing 100 mg of pepsin (PM, Worthington Biochemical Corp.) and incubating the suspension with constant stirring at 4° for 18 hr. Following incubation, the viscous digestion mixture was clarified by centrifugation at 50,000*g* for 1 hr at 4°, and collagen was precipitated from the clarified solution by the addition of crystalline NaCl to a concentration of 0.9 M. The resulting precipitate was retrieved by centrifugation at 50,000*g* for 1 hr at 4° and redissolved in 1.0 M NaCl (pH 7.5, 0.05 M Tris). The collagen solution was then dialyzed against a large volume of 0.5 M acetic acid at 4° and further purification was achieved as previously described for acid-soluble bone collagen (Miller *et al.*, 1967).

Chromatography of Pepsin-Solubilized Cartilage Collagen. During the chromatographic procedures used in the present study column effluents were monitored, recorded, and collected as described previously (Miller *et al.*, 1969) with the exception that DB-GT spectrophotometers (Beckman Instruments, Inc.) were employed in column monitoring. Carboxymethyl cellulose (CM-cellulose) chromatography of the pepsin-solubilized collagen after denaturation was performed on 1.8 × 10 cm columns of CM-cellulose (Whatman CM-32, microgranular, capacity 1.0 mequiv/g) employing buffers containing 1.0 M urea as described previously (Miller, 1971a). Aliquots of the material eluted from CM-cellulose columns as well as pepsin-solubilized collagen which had not been chromatographed on CM-cellulose were dissolved in 1.0 M $CaCl_2$ (pH 7.5, 0.05 M Tris), warmed to 40° for 15 min to ensure denaturation, and chromatographed at a flow rate of 16 ml/hr on a calibrated 2.0 × 265 cm column of agarose beads (Bio-Gel A-1.5m, 200–400 mesh, Bio-Rad Laboratories) for molecular weight determinations (Piez, 1968; Miller *et al.*, 1969).

CNBr Cleavage. Cleavage at the methionyl residues of both insoluble cartilage collagen and the pepsin-solubilized material with CNBr was achieved in 70% formic acid as previously described (Miller *et al.*, 1971; Miller, 1971b). Following the 4-hr digestion period, the reaction mixtures were diluted tenfold with distilled water and immediately lyophilized.

Chromatography of CNBr Peptides. Initial resolution of the CNBr peptides derived from insoluble cartilage collagen and pepsin-solubilized material was achieved on a 3.5 × 45 cm column of Bio-Gel P-2 (100–200 mesh, Bio-Rad Laboratories) equilibrated with 0.1 N acetic acid. Approximately 200 mg of lyophilized peptides was dissolved in 10 ml of 0.1 M acetic acid for application to the column. Two fractions of the P-2 eluent, representing the excluded volume and the total fluid volume of the column, were retained for further fractionation.

The larger peptides appearing in the excluded volume of the P-2 eluent were rechromatographed on a 2.5 × 10 cm column of CM-cellulose. The column was equilibrated with starting buffer (0.02 M sodium citrate, adjusted to pH 3.6 with citric acid and containing 0.01 M NaCl) and the peptides were dissolved in this buffer for application to the column. Chromatography was performed by means of a linear salt gradient established by 1000 ml of starting buffer and 1000 ml of limit buffer (starting buffer containing 0.16 M NaCl) in a constant-level device at a flow rate of 200 ml/hr and a column temperature of 42°. Peptides appearing in various fractions of the CM-cellulose eluent were desalted on Bio-Gel P-2 (column described above). Further purification and resolution of the peptides in each fraction were achieved by rechromatography on a 1.5 × 90 cm column of Bio-Gel P-6 (100–200 mesh, Bio-Rad Laboratories) equilibrated with 0.1 M acetic acid and operated at a flow rate of 50 ml/hr or by rechromatography on agarose beads as described above. In one instance, peptides eluted from the CM-cellulose column were further resolved by rechromatography on phosphocellulose under conditions described in the legend for Figure 7.

The smaller CNBr peptides eluted from the P-2 column were rechromatographed on a 1.0 × 8.0 cm column of phosphocellulose (Whatman, floc, capacity 7.4 mequiv/g). The column was equilibrated with starting buffer (0.001 M sodium acetate, adjusted to pH 3.8 with acetic acid) and the peptides were dissolved in this buffer for application to the column. Elution was achieved using a linear salt gradient provided by 200 ml of starting buffer and 200 ml of limit buffer (starting buffer containing 0.1 M NaCl) in a constant-level device at a flow rate of 50 ml/hr and a column temperature of 42°. One of the peptides chromatographed in this system was strongly retained by the column and was eluted by washing the column with starting buffer containing 0.3 M NaCl after elution with appropriate quantities of the gradient.

Amino Acid Analyses. All samples were prepard for hydrolysis and hydrolyzed at 110° for 24 hr in constant-boiling

TABLE I: Amino Acid Composition of Insoluble Cartilage Collagen and Some of the Cyanogen Bromide Peptides Derived Therefrom.

Amino Acid	Insoluble Collagen Residues/1000	Residues/Peptide[a]				
		14	7[b]	7–14	15	7-P
3-Hydroxyproline	2.0		1	1 (1.1)		1 (1.0)
4-Hydroxyproline	100		8	8 (7.8)		8 (7.9)
Aspartic acid	46	1 (1.1)	2	3 (3.0)		2 (2.1)
Threonine	28	1 (0.9)	1	2 (2.1)		1 (1.0)
Serine	29	1 (0.9)	1	2 (2.2)		1 (0.9)
Glutamic acid	93	2 (2.0)	2	4 (4.1)		2 (2.0)
Proline	108	2 (2.1)	8	10		8 (8.2)
Glycine	320	3 (3.0)	14	17		14
Alanine	100	2 (2.0)	2	4 (4.2)	1 (1.0)	1.0 (1.4)
Valine	18					
Methionine	13			1 (0.8)		
Isoleucine	8.8	1 (0.9)	2	3 (3.0)		2 (2.0)
Leucine	29	1 (0.9)		1 (0.9)		
Tyrosine	2.8	1 (1.1)		1 (1.1)		
Phenylalanine	14	1 (1.0)		1 (1.0)		
Hydroxylysine	21	1 (1.0)		1 (0.9)		
Histidine	2.0					
Lysine	13					
Arginine	52	1 (1.0)	2	3 (3.0)	1 (1.0)	2 (2.0)
Homoserine		1 (0.9)	1	1 (1.0)		
Total	1000	19	44	63	2	42

[a] Residues per peptide expressed to the nearest whole number. Actual calculated values are listed where less than ten residues are present. [b] Data from Miller (1971c).

6 N HCl as previously described (Miller *et al.*, 1969). Analyses were performed on an automatic amino acid analyzer (Model 119, Beckman Instruments, Inc.) equipped with an automatic sample injector (Model 138, Beckman Instruments, Inc.), four buffer-change modules, and a single column containing sulfonated polystyrene beads (Resin M-82, Beckman Instruments, Inc., Munich Division) with resin bed dimensions of 0.9 × 60 cm. Elution and resolution of all amino acids were achieved by sequential use of four buffers delivered to the column at a flow rate of 60 ml/hr. The column temperature was maintained at 51° throughout each run and ninhydrin was delivered to the column effluent at a rate of 30 ml/hr. The compositions of the buffers and the intervals throughout which they are employed during each run are as follows: buffer A, 0.2 N (Na^+) sodium citrate, pH 2.95, 0–55 min; buffer B, 0.2 N (Na^+) sodium citrate, pH 3.2, 56–120 min; buffer C, 0.2 N (Na^+) sodium citrate, pH 4.1, 121–165 min; buffer D, 0.3 N (Na^+) sodium citrate containing 0.9 M NaCl, pH 6.1, 166–320 min; buffer A for reequilibration, 321–360 min.

In calculating amino acid chromatograms, hydrolytic losses of threonine, serine, and tyrosine, and incomplete release of valine were corrected for by application of factors previously determined for collagen (Piez *et al.*, 1960).

Results

Preparation of Collagen. During the preparation of the insoluble collagen used as the starting material in the present studies, the nondialyzable material contained in each extract was submitted to hydrolysis and amino acid analysis.In accordance with previous results (Miller and Matukas, 1969) it was observed in the present studies that extraction of fresh cartilage slices from normal animals with 1.0 M NaCl at neutral pH and 0.5 M acetic acid was totally ineffective in solubilizing cartilage collagen as judged by the absence of hydroxyproline and hydroxylysine in the extracts. Nevertheless, amino acid analyses (Table I) of the residue remaining after these extractions indicated that the extraction procedures were quite effective in removing noncollagenous contaminants from the tissue and the amino acid composition of the insoluble collagen is very similar to that previously determined for purified α1(II) chains from chick cartilage (Miller, 1971a).

CNBr Peptides of Insoluble Cartilage Collagen. Further characterization of the insoluble cartilage collagen was obtained in an examination of the CNBr peptides liberated from the material during cleavage at methionyl residues. In accordance with previous results (Miller *et al.*, 1971) where this technique was applied to insoluble collagen of human epiphyseal cartilage, recovery of the total chick cartilage collagen as soluble CNBr peptides was essentially quantitative and less than 2% of the total hydroxyproline in the sample remained as insoluble material after the 4-hr digestion period.

Figure 1 depicts a representative chromatogram of the larger CNBr peptides from insoluble cartilage collagen chromatographed on CM-cellulose. The two large peaks appearing in the eluent immediately following initiation of the gradient are comprised largely of acidic polysaccharide molecules and following hydrolysis the predominan tninhydrin-positive components of these peaks are the amino hexoses, glucos-

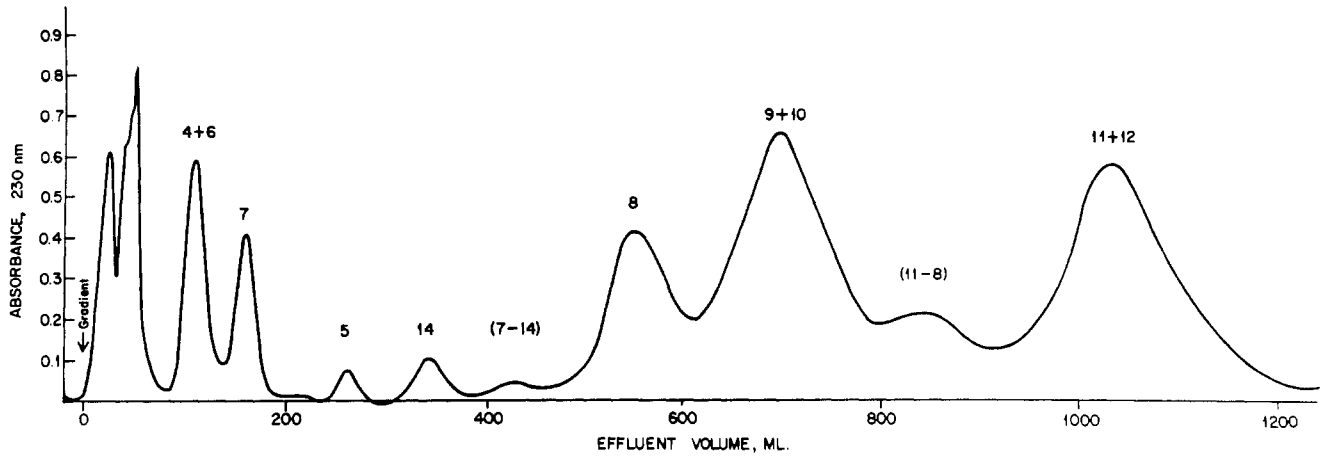

FIGURE 1: Elution pattern of the larger CNBr peptides derived from insoluble cartilage collagen when chromatographed on a 2.5 × 10 cm column of CM-cellulose under conditions described in the text. The total weight of the sample chromatographed in this instance was 250 mg.

amine and galactosamine. As indicated in the figure, all of the collagen-derived CNBr peptides chromatographed in this system exhibit some degree of retention under the conditions employed and the peptides are numbered according to the system previously adopted (Miller, 1971c). For the most part, each of the peptides chromatographed in this system is identical to those previously observed as CNBr cleavage products of soluble α1(II) from the sternal cartilages of lathyritic chicks (Miller, 1971c). The sole exception to this observation is the appearance in the cleavage products of insoluble cartilage collagen of peptide 14 (eluted at an effluent volume of approximately 350 ml; Figure 1), a peptide which was not detected in the cleavage products of soluble cartilage collagen. The amino acid composition of peptide 14, listed in Table I, indicates that it is derived from a nonhelical portion of the cartilage collagen molecule since it contains only 3 glycyl residues in a total sequence of 19 amino acids. Although peptide 14 has an unusual composition with respect to most of the regions of the collagen molecule it was possible to demonstrate that it is an integral part of the α1(II) chain since approximately 15% of the peptide recovered in several CNBr digests remained linked to an adjacent peptide, peptide 7, due to incomplete cleavage at the methionyl residue joining the peptides. The elution position of the peptide resulting from incomplete cleavage, designated (7–14), is shown in Figure 1 and its amino acid composition is given in Table I along with peptide 7 as a reference.

Figure 2 illustrates the elution pattern of the smaller CNBr peptides derived from insoluble cartilage collagen when chromatographed on phosphocellulose. The peptides designated 1, 2, and 3 are identical with those observed in the cleavage products of soluble α1(II) (Miller, 1971c.) However, the strongly retained peptide, designated peptide 15, was not observed in soluble cartilage collagen. Amino acid analyses of the latter peptide revealed it to be a dipeptide containing alanine and arginine (Table I) which therefore must represent the COOH-terminal region of the cartilage collagen molecule as prepared in the present study. On the basis of amino acid analyses of the peptides isolated during phosphocellulose chromatography, each of the peptides are recovered in equivalent amounts.

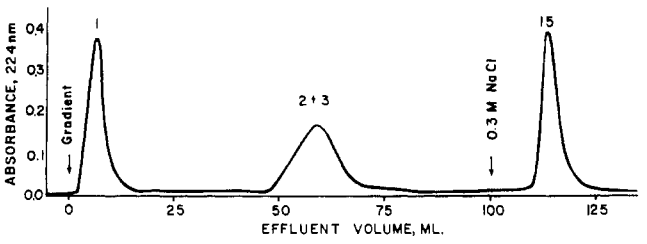

FIGURE 2: Elution pattern of the smaller CNBr peptides derived from insoluble cartilage collagen when chromatographed on a 1.0 × 8.0 cm column of phosphocellulose under conditions described in the text. Elution with starting buffer containing 0.3 M NaCl was initiated at the point indicated by the arrow. The sample applied to the column represents the smaller peptides isolated from 250 mg of CNBr peptides.

Characterization of Pepsin-Solubilized Cartilage Collagen. Incubation of the insoluble cartilage collagen with pepsin under the conditions cited above routinely resulted in the recovery of 60–70% of the weight of the starting material as purified soluble cartilage collagen. Incubation with pepsin at higher temperatures such as 8 or 12° did not appreciably increase the yield of soluble collagen; therefore a lower temperature was used in order to avoid any possibility of approaching conditions under which the collagen helix might begin to unfold.

A CM-cellulose chromatogram illustrating the elution pattern of denatured pepsin-solubilized cartilage collagen is presented in Figure 3. The chromatogram is quite similar to that previously observed for α1(II) chains extracted from the sternal cartilages of lathyritic animals (Miller, 1971a), although the heterogeneity observed in the trailing edge of the peak is considerably more pronounced in this instance. Amino acid analyses of fractions taken throughout the peak showed no significant differences and the results corresponded to previously published values for the amino acid composition of α1(II) (Miller, 1971a). As will be indicated below, the chromatographic heterogeneity observed in the α1(II) chains derived from pepsin-solubilized cartilage collagen may be ascribed to chemical heterogeneity introduced by activity of the enzyme.

The molecular weight distribution of the components in denatured pepsin-solubilized cartilage collagen was examined by molecular sieve chromatography on a calibrated column of agarose beads. A chromatogram illustrating the elution profile of material not previously chromatographed on CM-cellulose is presented in Figure 4. The majority of the material applied to the column chromatographed as a single peak with

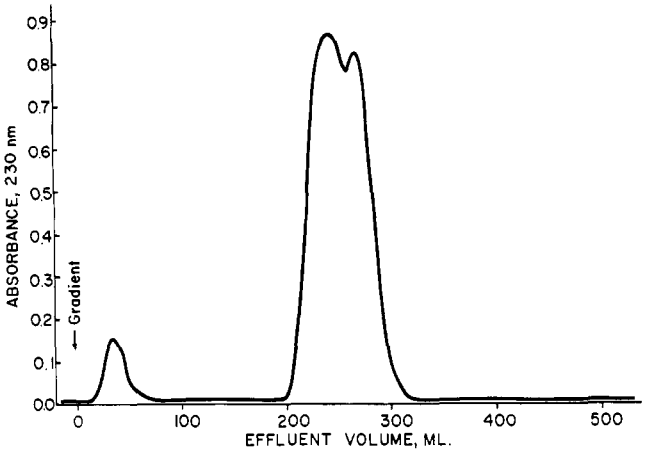

FIGURE 3: CM-cellulose elution pattern of 100 mg of denatured pepsin-solubilized cartilage collagen. Chromatography is performed on a 1.8 × 10 cm column under conditions identical with those previously described (Miller, 1971a).

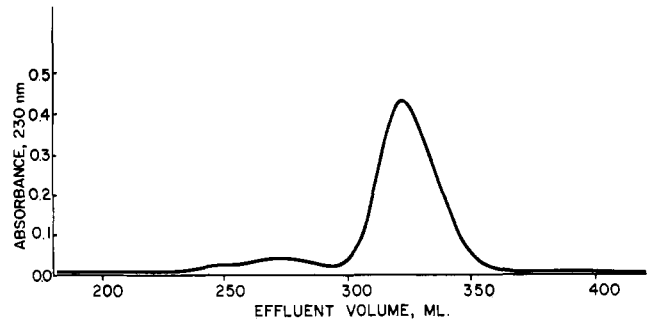

FIGURE 4: Elution pattern observed during chromatography of 20 mg of denatured pepsin-solubilized cartilage collagen on a 2.0 × 265 cm column of agarose beads under conditions described in the text.

an elution volume of 320 ml corresponding to that of α chains with a molecular weight of 95,000. Identical results were obtained when chains recovered from CM-cellulose chromatography were chromatographed in the same system. In the system employed, β components are eluted at 240 ml and no evidence for the presence of these or higher molecular weight components was obtained in the pepsin-solubilized cartilage collagen.

Collagen recovery after chromatography on CM-cellulose and agarose columns was estimated by weighing the desalted, lyophilized material eluted from the columns. In both types of chromatography, the material recovered in this manner represented between 85 and 90% of the sample originally applied to the column.

CNBr Peptides of Pepsin-Solubilized Cartilage Collagen. CNBr peptides derived from the α1(II) chains of pepsin-solubilized cartilage collagen after CM-cellulose chromatography were examined in the manner described above for CNBr peptides derived from insoluble cartilage collagen in order to determine the extent of enzyme activity on the cartilage collagen molecule.

A CM-cellulose chromatogram of the larger CNBr peptides from pepsin-solubilized cartilage collagen is presented in Figure 5. Although this elution pattern is similar to that obtained when CNBr peptides derived from insoluble cartilage collagen are chromatographed in the same system (Figure 1), two differences are readily apparent. Namely, peptide 14 is not present in the CNBr cleavage products of pepsin-solubilized collagen and the apparent yield of peptide 7 is considerably reduced. In addition to these obvious differences, rechromatography of the region normally containing peptides 6 and 4 on Bio-Gel P-6 (example shown in Figure 6, top) revealed that peptide 4 was also absent from the CNBr cleavage products of pepsin-solubilized cartilage collagen (Figure 6, bottom). Furthermore, as also shown in the lower portion of Figure 6, this region also contained material with a molecular weight somewhat greater than peptide 6, designated peptide (7-P). Peptide 6 was resolved from peptide (7-P) by rechromatography on phosphocellulose as depicted in Figure 7. The amino acid analysis of peptide (7-P) is listed in Table I indicating that it is derived from peptide 7 but differs from the latter in that all molecules lack homoserine and some of them lack one of the alanyl residues.

Characterization of the remaining peptides depicted in the chromatogram presented in Figure 5 indicated that they are identical with those previously observed in preparations of soluble α1(II) (Miller, 1971c) and those prepared from insoluble cartilage collagen in the present study.

The smaller CNBr peptides derived from pepsin-solubilized cartilage collagen were rechromatographed on phosphocellulose under conditions identical with those utilized for the smaller peptides from insoluble cartilage collagen. The results of this study indicated that of the four peptides (1, 2, 3, and 15) normally present in this fraction, only peptides 2 and 3 were present in the pepsin-solubilized collagen.

Discussion

In contrast to several previous studies on the CNBr cleavage products derived from α chains present in soluble collagen preparations from a variety of tissues in a number of species, the present studies have been performed utilizing a partially purified insoluble cartilage collagen preparation as the starting material. In the course of these investigations it was noted that the CNBr cleavage products of insoluble cartilage collagen are, for the most part, qualitatively and quantitatively identical with those previously observed in CNBr digests of purified α1(II) prepared from soluble cartilage collagen (Miller, 1971c). However, the present studies have demonstrated the existence of two additional sequences in the α1(II) chain represented by the CNBr peptides designated 14 and 15, and comprising a total of 21 amino acids.

In addition, data have been presented indicating that a substantial proportion of cartilage collagen which is not normally extractable as native collagen may be solubilized as native collagen by incubating the insoluble material with pepsin. Characterization of the pepsin-solubilized cartilage collagen with respect to chain composition, molecular weight of the component α chains, and CNBr cleavage products further demonstrated that the proteolytic activity of pepsin on the cartilage collagen molecule is restricted to relatively short sequences represented by the CNBr peptides, 1, 4, 14, 15, and the COOH-terminal portion of peptide 7.

These results indicate that the cited sequences do not participate in collagen helix formation and that they occur at the extremities of the α1(II) chains comprising the cartilage collagen molecule. These data have been confirmed by additional studies designed to determine the order of all the CNBr peptides in the α1(II) chain and it is known, for instance, that the sequences provided by peptides 1 and 4 comprise the first 15 amino acids at the NH_2-terminal end of the

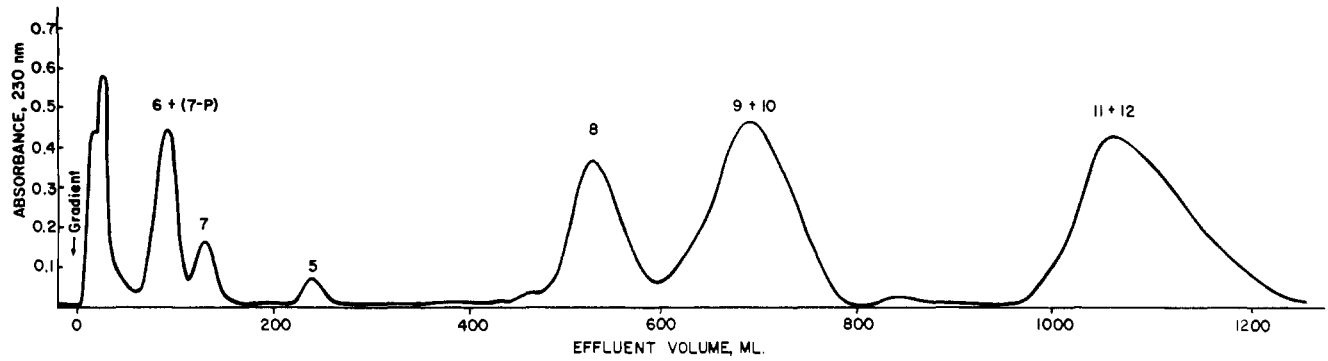

FIGURE 5: Elution pattern of the larger CNBr peptides derived from the α1(II) chains of pepsin-solubilized cartilage collagen. The total weight of the sample chromatographed in this instance was 200 mg.

α1(II) chain (E. J. Miller, D. L. Woodall, and M. S. Vail, manuscript in preparation). Moreover, the present studies allow the localization of peptides 7, 14, and 15, in the α1(II) chain. Peptide 15 is clearly derived from the carboxy-terminal end of the chain since it lacks homoserine and it has been demonstrated that the sequence represented by peptide 14 lies adjacent to the sequence represented by peptide 7 in the chain. The absence of both peptides 14 and 15 as well as the loss of a homoseryl residue and partial loss of an alanyl residue from peptide 7 in the pepsin-solubilized chain clearly establishes that peptide 14 is positioned on the carboxy-terminal side of peptide 7 and indicates that the order of the CNBr peptides in the carboxy-terminal region of the α1(II) chain is 7-14-15. These results indicate that the protease-sensitive nonhelical region located at the COOH-terminal region of the cartilage collagen molecule and previously detected by electron microscope studies on segment-long-spacing fibers prepared from control and pepsin-treated cartilage collagen (Stark *et al.*, 1972) comprises a sequence of 23 amino acids. Although the electron microscope studies suggested that the nonhelical sequence at the COOH-terminal region might be somewhat longer than determined in the present study, the apparent discrepancy in these results may be explained by the nature of the nonhelical region itself in which the contribution of each amino acid residue to the axial length of the molecule would be greater than in helical portions of the molecule. Alternatively, the explanation may be attributed to the fact that previous studies with pepsin were performed at 18° at which temperature a moderate increase in the tendency for collagen chains to unfold combined with increased activity on the part of the enzyme may have contributed to cleavage at a point further removed from the COOH-terminal region.

The results discussed above also suggest that failure to detect peptides 14 and 15 in the CNBr cleavage products of α1(II) prepared from soluble cartilage collagen (Miller, 1971c) resulted from nonspecific proteolytic activity during extraction and purification of several preparations of the material. Moreover, it is likely that the COOH-terminal tyrosyl residue previously detected in α1(II) chains prepared from soluble cartilage collagen (Miller, 1971c) is the tyrosyl residue present in the sequence of peptide 14. Evidence for the occurrence of nonspecific proteolytic degradation of the nonhelical NH_2-terminal portion of the collagen molecule during extraction and purification has previously been reported in studies on rat skin (Bornstein, 1969) and chick skin (Kang *et al.*, 1969) collagens. More recently, the presence of a nonhelical region at the COOH-terminal end of the collagen molecule with similar susceptibility to nonspecific proteolytic degradation has been detected in studies on calf skin collagen (Stark *et al.*, 1971). The present study has chemically defined the nonhelical region occurring at the COOH-terminal region of the cartilage collagen molecule with the chain composition, $[\alpha 1(II)]_3$, and indicates that a nonhelical

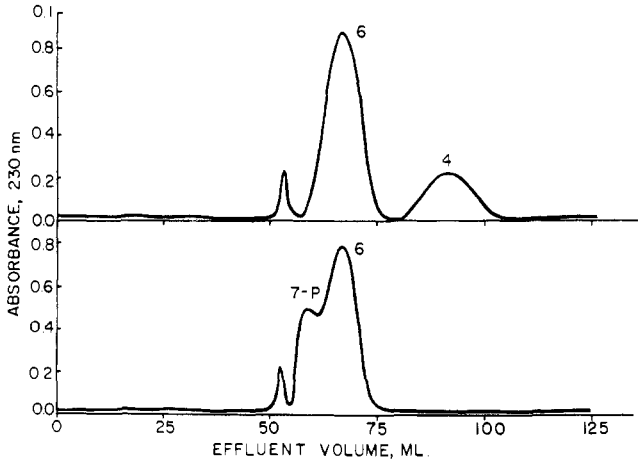

FIGURE 6: Bio-Gel P-6 rechromatography of the CM-cellulose eluent containing peptides 6 and 4 (effluent volume, 100–150 ml, Figure 1) illustrating the resolution of these peptides derived from insoluble cartilage collagen (top). Bio-Gel P-6 rechromatography of a similar portion of the CM-cellulose eluent (Figure 5) illustrating the absence of peptide 4 and the appearance of peptide (7-P) in the CNBr cleavage products derived from pepsin-solubilized cartilage collagen (bottom).

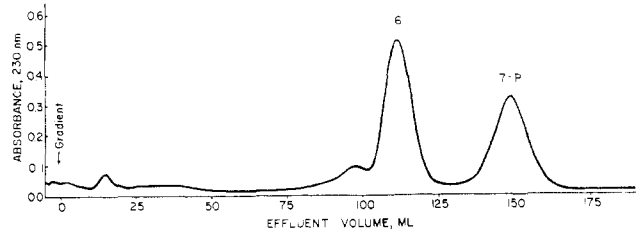

FIGURE 7: Phosphocellulose chromatogram illustrating the resolution of peptides 6 and (7-P). Chromatography was performed on a 1.0 × 8.0 cm column of phosphocellulose and elution was achieved in 0.001 M (Na^+) sodium acetate (pH 3.8) using a linear salt gradient of NaCl from 0 to 0.1 M. The total volume of the gradient was 400 ml.

COOH-terminal segment is common to all collagen molecules, regardless of tissue origin and type. In view of the susceptibility of this region to nonspecific proteolytic activity it is possible that virtually any method employed to isolate collagen will result in some modification of the COOH-terminal region and that our information with respect to the nature of this portion of the molecule is as yet incomplete. It is of interest to note that the nonhelical region from the COOH-terminal end of the α1(I) chain of calf skin collagen comprises 21 amino acids (Rauterberg *et al.*, 1972) and exhibits many of the compositional features indicated in the present study for the 21 amino acid sequence represented by peptides 14 and 15.

With respect to the mechanism whereby incubation with pepsin profoundly alters the solubility properties of cartilage collagen, there is at present insufficient data to evaluate all possible modes of action. However, it is known that the hydroxylysyl residue of peptide 4 participates along with one of the hydroxylysyl residues of peptide 9 to form one site of intermolecular cross-linking (Miller, 1971b). It is likely, therefore, that proteolytic degradation of the sequence represented by peptide 4 serves in effect to eliminate this site of intermolecular cross-linking, thus allowing solubilization of the collagen as monomeric molecules. Whether or not intermolecular cross-linking also occurs in the sequence represented by peptide 14 is not known at present.

It seems appropriate also to comment on the general applicability of the methodology presented in this study. It has been shown that substantial quantities of cartilage collagen can be prepared in soluble form from the sternal cartilages of normal chickens by incubation with a proteolytic enzyme whose activity is restricted to short sequences at the extremities of the cartilage collagen molecule. Moreover, prior removal of the majority of the proteoglycan components of the tissue by relatively simple extraction procedures eliminates the necessity to separate the solubilized collagen from acidic mucopolysaccharide molecules by DEAE-cellulose chromatography as previously indicated (Miller, 1971a) when collagen is extracted directly from the cartilages of lathyritic animals. Further results have demonstrated that the methodology employed here is equally effective for cartilages of mammalian origin provided that acidic proteoglycans are first removed by more potent solvents (Sajdera and Hascall, 1969). The methodology allows, then, the preparation of substantial quantities of soluble cartilage collagen in native form from species in which enhancement of collagen solubility by the administration of lathyrogens is either not practical or impossible. The only disadvantage stemming from use of this technique is derived from the loss of short NH_2- and COOH-terminal sequences. However, from the above discussion it is clear that considerable degradation in these regions can occur even during extraction procedures not utilizing additive enzymes.

References

Bornstein, P. (1969), *Biochemistry 8*, 63.

Kang, A. H., Piez, K. A., and Gross, J. (1969), *Biochemistry 8*, 3648.

Miller, E. J. (1971a), *Biochemistry 10*, 1652.

Miller, E. J. (1971b), *Biochem. Biophys. Res. Commun. 45*, 444.

Miller, E. J. (1971c), *Biochemistry 10*, 3030.

Miller, E. J., Epstein, E. H., Jr., and Piez, K. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 1024.

Miller, E. J., Lane, J. M., and Piez, K. A. (1969), *Biochemistry 8*, 30.

Miller, E. J., Martin, G. R., Piez, K. A., and Powers, M. J. (1967), *J. Biol. Chem. 242*, 5481.

Miller, E. J. and Matukas, V. J. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1264.

Piez, K. A. (1968), *Anal. Biochem. 26*, 305.

Piez, K. A., Weiss, E. and Lewis, M. S. (1960), *J. Biol. Chem. 235*, 1987.

Rauterberg, J., Fietzek, P., Rexrodt, F., Becker, V., Stark, M., and Kühn, K. (1972), *FEBS (Fed. Eur. Biol. Soc.) Lett. 21*, 75.

Sajdera, S. W., and Hascall, V. C. (1969), *J. Biol. Chem. 244*, 77.

Stark, M., Miller, E. J., and Kühn, K. (1972), *Eur. J. Biochem. 27*, 192.

Stark, M., Rauterberg, J., and Kühn, K. (1971), *FEBS (Fed. Eur. Biol. Soc.) Lett. 13*, 101.

Strawich, E., and Nimni, M. E. (1971), *Biochemistry 10*, 3905.

Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J. (1970), *Biochemistry 9*, 4993.

Toole, B. P., Kang, A. H., Trelstad, R. L., and Gross, J. (1972), *Biochem. J. 127*, 715.