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Isolation and Characterization of the Cyanogen Bromide Peptides from the α1(II) Chain of Bovine and Human Cartilage Collagen†

Edward J. Miller* and Linda G. Lunde

ABSTRACT: Insoluble collagen prepared from three mammalian cartilages (bovine nasal septum, bovine articular cartilage, and human articular cartilage) was cleaved with cyanogen bromide (CNBr). The solubilized peptides which in each instance accounted for over 95% of the tissue collagen were isolated by molecular sieve and ion-exchange chromatography and characterized with respect to molecular weight and amino acid composition. Twelve and ten peptides were isolated as CNBr cleavage products of the bovine and human cartilage collagens, respectively. For each species, the isolated peptides account for virtually the entire length of an α1(II) chain. The data indicate that identical peptides are derived from both bovine cartilages although peptides from articular cartilage collagen exhibit slightly less hydroxylation of lysyl residues. The peptides from bovine cartilage collagen are very similar to those from human cartilage collagen and exhibit only small differences in amino acid composition. Furthermore, all of the peptides characterized in the present study are clearly recognizable as homologs of those previously isolated from chick α1(II), demonstrating a close structural homology between the α1(II) chains in the cartilages of various species.

Previous studies on the collagen in several cartilaginous tissues have indicated that the cartilage collagen molecule is comprised of three identical α chains. The latter have been designed α1(II) chains to indicate their derivation from a genetic locus distinct from the loci directing the synthesis of α1(I) and α2 chains found in the collagen of bone, skin, tendon, and other tissues (Miller and Matukas, 1969; Trelstad *et al.*, 1970; Miller, 1971a; Miller *et al.*, 1971; Strawich and Nimni, 1971; Toole *et al.*, 1972). Thus, the cartilage collagen molecule with the chain composition $[\alpha 1(II)]_3$ represents a striking example of tissue specificity in the biosynthesis of this macromolecule.

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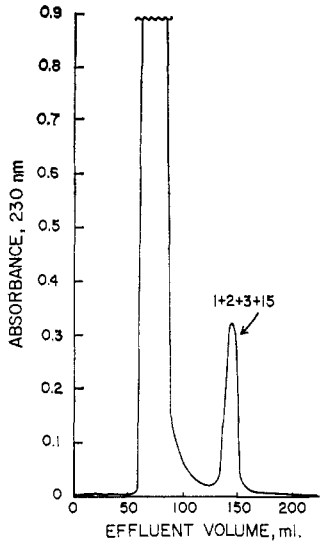

FIGURE 1: Elution pattern of the CNBr peptides derived from 200 mg of bovine nasal cartilage collagen when chromatographed on Bio-Gel P-2. Patterns obtained when similar amounts of CNBr peptides from bovine or human articular cartilage collagen were chromtographed were identical with that depicted here. Fractions chosen for further chromatography comprised the elution volumes between 55–100 and 125–160 ml.

Collagen derived from chick cartilages has been extensively characterized with respect to chain composition, amino acid composition of the α1(II) chain, and physicochemical properties of the intact molecule (Trelstad *et al.*, 1970; Miller, 1971a; Toole *et al.*, 1972; Stark *et al.*, 1972; Igarashi *et al.*, 1973). In addition, the cyanogen bromide (CNBr) cleavage products accounting for all the amino acids and molecular weight of chick α1(II) have been isolated and characterized (Miller, 1971b, 1972). The linear order of these peptides along the α1(II) chain has been established (Miller, 1972; Miller *et al.* 1973).

Studies on mammalian cartilage collagen have thus far been limited to isolation of two of the CNBr peptides from the α1(II) chain of human growth plate cartilage (Miller *et al.*, 1971) and isolation of the α1(II) chain of bovine articular cartilage after preincubation of the tissue with papain (Strawich and Nimni, 1971). We report here the isolation and characterization of the CNBr peptides derived from the α1(II) chain of bovine nasal septum and articular cartilages as well as human articular cartilage. In view of the extreme insolubility of mammalian cartilage collagen (Miller *et al.*, 1969) our studies have proceeded by employing direct CNBr cleavage of insoluble collagen preparations allowing characterization of these collagens at the level of the peptides thus released.

The results of these studies indicate that all of the collagen in the tissues under investigation may be accounted for as molecules with the chain composition $[\alpha 1(II)]_3$. The data further indicate a high degree of structural homology between chick and mammalian cartilage collagens since all of the CNBr peptides derived from mammalian α1(II) are clearly recognizable as homologs of CNBr peptides previously isolated from chick α1(II) (Miller, 1971b, 1972).

Materials and Methods

Sources and Preparation of Collagen. Nasal septa from ~2-year old cattle were obtained at a local slaughterhouse and freed of adhering tissue and perichondrium. Articular cartilage was shaved with a scalpel blade from the distal articular surfaces of the femora and proximal articular surfaces of the tibiae of the same animals. Human articular cartilage was obtained at autopsy in the same manner from the knees (including patellar, femoral, and tibial surfaces) of two females aged 10 and 12 years who showed normal skeletal development and who had died of causes unrelated to connective tissue disease. None of the articular cartilage samples showed evidence of fibrillation or degeneration.

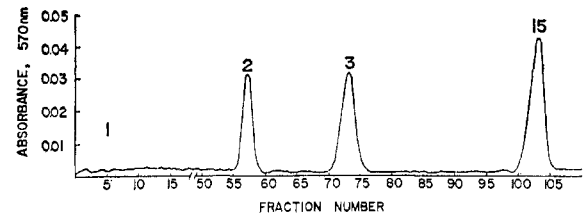

FIGURE 2: Elution pattern of the smaller CNBr peptides derived from bovine nasal cartilage collagen when chromatographed on Hi-Rex DC-1A. The conditions of chromatography are described in the text. Identical patterns were obtained when the smaller peptides from bovine or human articular cartilage collagen were chromatographed in the same system.

Each type of cartilage was further diced with a scalpel blade into small cubes approximating 1 mm^3 in size. In order to remove proteoglycan molecules and other noncollagenous components of the cartilages, the small cubes of each preparation were subsequently extracted with constant stirring at room temperature with 15 vol of 4.0 M guanidinium chloride (absolute grade, Research Plus Laboratories) (pH 7.5)–0.05 M Tris, for 24 hr (Sajdera and Hascall, 1969). Following the extraction period, the cubes were thoroughly rinsed with distilled water and lyophilized. The insoluble residue thus obtained was employed as the starting material for subsequent studies.

CNBr Cleavage. Cleavage at methionyl residues of the insoluble cartilage collagen preparations was performed in 70% formic acid as previously described (Miller *et al.*, 1971). Following the 4-hr cleavage period the reaction mixtures were centrifuged at 5000*g* for 30 min to remove any remaining particulate matter. The supernatant fluid containing solubilized cartilage collagen peptides was then diluted tenfold with distilled water and lyophilized.

Chromatography of CNBr Peptides. CNBr peptides from each preparation of cartilage collagen were initially resolved into two major fractions by chromatography on Bio-Gel P-2 (Bio-Rad Laboratories) as previously described (Miller, 1972).

The smaller CNBr peptides eluting in the total fluid volume of the P-2 column were collected after lyophilization by redissolving in water. These peptides were then resolved on a 0.9 × 55 cm column of Hi-Rex DC-1A (Pierce Chemical Co.) at 49° using a Beckman 120C automatic amino acid analyzer equipped with a split stream device. After application of the peptides in 1.0 ml of water, the column was eluted at a flow rate of 80 ml/hr with a gradient formed by a nine-chambered varigrad. The varigrad chambers were prepared as follows: chambers 1, 2, and 3 each contained 80 ml of sodium citrate buffer, pH 2.91 (Piez and Morris, 1960); chamber 4 contained 70 ml of pH 2.91 buffer plus 10 ml of 0.4 M sodium citrate; chamber 5 contained 40 ml each of pH 2.91 buffer and 0.4 M sodium citrate; chamber 6 contained 20 ml of pH 2.91 buffer plus 60 ml of 0.4 M sodium citrate; and chambers 7, 8, and 9 each contained 80 ml of 0.4 M sodium citrate. Aliquots of the appropriate peptide fractions eluted from the Hi-Rex column were hydrolyzed without removal of salts by addition of an equal volume of concentrated HCl and heating in evacuated sealed tubes at 108° for 24 hr.

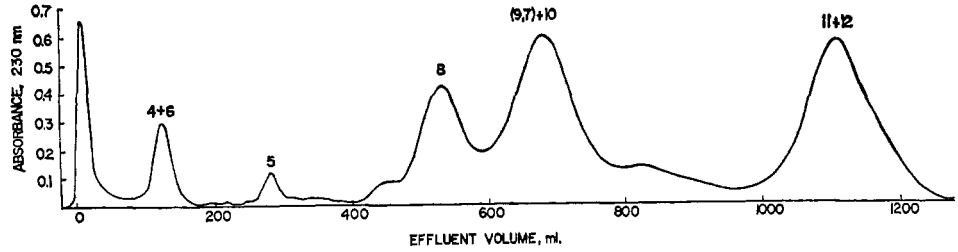

FIGURE 3: CM-cellulose chromatogram illustrating the resolution of the larger CNBr peptides derived from bovine nasal cartilage collagen. The conditions for chromatography have been described (Miller, 1972).

The larger peptides eluted in the excluded volume of the P-2 eluent were further resolved by rechromatography on a 2.5 × 10 cm column of carboxymethylcellulose (CM-cellulose, Whatman CM-32, microgranular, capacity 1.0 mequiv/g) employing conditions identical with those previously described for chromatography of the larger CNBr peptides of chick cartilage collagen (Miller, 1972). Peptides eluting in the CM-cellulose eluent were desalted 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. Additional purification and resolution of the peptides in each CM-cellulose fraction were obtained by rechromatography either on a 1.5 × 90 cm column of Bio-Gel P-6 (100–200 mesh, Bio-Rad Laboratories) equilibrated with 0.1 N acetic acid and used at a flow rate of 50 ml/hr, or by rechromatography on agarose beads (see below).

Molecular Weight Determinations. Molecular weights of the larger CNBr peptides observed in this study were estimated by chromatography at a flow rate of 10 ml/hr on a calibrated 1.5 × 110 cm column of agarose beads (Bio-Gel A-1.5m, 200–400 mesh, Bio-Rad Laboratories) as previously described by Piez (1968).

Amino Acid Analyses. With the exception of the hydrolysis conditions described above for the smaller CNBr peptides, all peptide samples were hydrolyzed under nitrogen at 108° for 24 hr in constant-boiling 6 N HCl. Amino acid analyses were subsequently performed on an automatic amino acid analyzer equipped with a sample injector (Model 119, Beckman Instruments, Inc.) as previously described (Miller, 1972).

In calculating amino acid chromatograms, previously determined correction factors were applied for the destruction of threonine, serine, and tyrosine, and incomplete release of valine during hydrolysis (Piez *et al.*, 1960).

Results

Isolation of CNBr Peptides of Bovine and Human Cartilage Collagen. Recovery of total bovine and human cartilage collagen as soluble CNBr peptides was estimated by weighing the lyophilized peptide mixtures. In each case (bovine nasal and articular cartilage collagen as well as human articular cartilage collagen) the weight of the lyophilized peptides exceeded 95% of the weight of the starting material.

Figure 1 depicts a representative chromatogram illustrating the initial resolution of the CNBr peptides[1] derived from bovine nasal cartilage collagen on Bio-Gel P-2. Similar chromatograms were obtained when CNBr peptide mixtures from bovine and human articular cartilage collagen were chromatographed in the same system. Indeed, our studies revealed that, with the exception of the extent of lysine hydroxylation, the CNBr peptides derived from bovine articular cartilage collagen are identical with those released from bovine nasal cartilage collagen. Therefore, the results presented here will be confined to the peptides from bovine nasal cartilage collagen and human articular cartilage collagen.

As illustrated in Figure 2, the smaller peptides (1, 2, 3, and 15) eluted in the total fluid volume of the P-2 column were subsequently resolved by cation-exchange chromatography on Hi-Rex DC-1A. These peptides occur as well in bovine and human articular cartilage collagen and chromatograms of these peptides from both sources are identical with the one shown in Figure 2 for bovine nasal cartilage collagen. It should also be noted that peptide 1 which elutes at approximately fraction 5 is not retained by the column and does not yield a 570-nm absorbance when allowed to react with ninhydrin. In light of the amino acid composition of peptide 1 (Tables I and II), these observations are in agreement with previous data on chick cartilage collagen indicating that peptide 1 may be identified as a dipeptide, pyrrolidonecarboxylic acid homoserine (Miller, 1971b, 1972; Miller *et al.*, 1973).

Figure 3 depicts a representative chromatogram illustrating the elution pattern of the larger CNBr peptides from bovine nasal cartilage collagen when chromatographed on CM-cellulose. The chromatogram closely resembles that obtained when the larger CNBr peptides from chick cartilage collagen are chromatographed in the same system (Miller, 1972). It differs from the latter in that peptides 7 and 14 are not observed in the reaction products of bovine nasal cartilage collagen. Moreover, the yield of peptide 4 in CNBr reaction mixtures of bovine nasal cartilage collagen is approximately one-tenth the anticipated amount as illustrated in Figure 4 where the resolution of peptides 6 and 4 on Bio-Gel P-6 is depicted.

The apparent absence of peptide 7 in CNBr digests of bovine nasal cartilage collagen is due to the absence of a methionyl residue in the bovine α1(II) chain relative to chick α1(II). This has been demonstrated by the isolation of peptide (9,7) which cochromatographs with peptide 10 on CM-cellulose (Figure 3). These peptides are readily separated by rechromatography on agarose as shown in Figure 5. The data presented in Table I for peptide (9,7) clearly indicate that it has a molecular weight and compositional features corresponding to the sum of peptides 9 and 7 from chick α1(II) (Miller, 1971b).

Peptides 4 and 14 are derived from short nonhelical sequences which occur respectively at the NH_2- and COOH-terminal regions of the cartilage collagen molecule (Miller, 1972; Miller *et al.*, 1973). Moreover, it is known that the hydroxylysyl residue of peptide 4 is one site of intermolecular

[1] The peptides characterized in the present study are assigned numbers indicative of their homology to the peptides previously characterized from chick cartilage collagen (Miller, 1971b, 1972). In those areas where a methionyl residue is missing in mammalian cartilage collagen, the peptide corresponding to two CNBr peptides of chick cartilage collagen is designated with both numbers separated by a comma (Miller *et al.*, 1969).

TABLE I: Amino Acid Composition[a] of CNBr Peptides from α1(II) of Bovine Nasal and Articular[c] Cartilage Collagen.

Amino Acid	1	2	3	4	5	6	8	(9,7)	10	11	12	15	Total CNBr Peptides
3-Hydroxyproline	0	0	0	0	0	0	0	2 (1.9)	0	0	0	0	2
4-Hydroxyproline	0	0	0	0	0	4 (4.2)	14	9 (8.9)	28	27	9 (9.1)	0	91
Aspartic acid	0	0	0	1 (1.1)	0	1 (0.9)	5 (5.0)	6 (6.0)	16	10	4 (4.1)	0	43
Threonine	0	0	0	0	0	0	3 (2.9)	3 (3.1)	7 (7.1)	8 (8.1)	1 (1.0)	0	22
Serine	0	0	0	0	0	1 (0.9)	3 (3.0)	5 (4.8)	8 (8.2)	5 (5.0)	4 (3.8)	0	26
Glutamic acid	1 (1.0)	0	0	2 (2.0)	0	4 (4.2)	16	8 (8.0)	27	22	7 (7.1)	0	87
Proline	0	0	1 (1.0)	0	2 (2.1)	6 (6.0)	18	20	39	35	8 (7.9)	0	129
Glycine	0	1 (1.0)	1 (1.0)	4 (4.2)	4 (4.0)	11	50	38	107	89	28	0	333
Alanine	0	0	0	3 (3.1)	3 (2.8)	2 (2.1)	14	6 (6.1)	38	30	5 (5.2)	1 (1.0)	102
Valine	0	1 (0.8)	0	0	0	1 (1.1)	2 (2.1)	1 (1.1)	6 (6.1)	4 (4.1)	2 (2.1)	0	17
Isoleucine	0	0	0	0	0	0	0	3 (2.8)	3 (2.9)	3 (2.8)	0	0	9
Leucine	0	0	0	0	0	0	8 (7.8)	3 (3.0)	6 (6.2)	7 (6.8)	2 (2.0)	0	26
Tyrosine	0	0	0	0	0	0	0	0	0	0	1 (1.0)	0	1
Phenylalanine	0	0	0	1 (1.0)	0	1 (0.9)	3 (3.1)	1 (1.0)	3 (3.0)	4 (4.0)	1 (1.1)	0	14
Hydroxylysine	0	0	0	1 (0.9)	0	0	3 (3.2)	2 (2.1)	7 (6.9)	7 (6.9)	3 (2.8)	0	23
Lysine	0	0	0	0	0	0	3 (2.9)	1 (1.0)	5 (4.8)	3 (3.1)	3 (3.0)	0	15
Histidine	0	0	0	0	0	0	0	1 (1.0)	0	0	1 (1.0)	0	2
Arginine	0	0	0	0	1 (1.0)	1 (1.0)	6 (6.1)	6 (6.0)	15	17	4 (4.0)	1 (1.0)	51
Homoserine	1 (1.0)	1 (0.9)	1 (0.9)	1 (1.0)	1 (1.0)	1 (1.0)	1 (0.9)	1 (1.0)	1 (0.9)	1 (1.0)	1 (0.9)	0	11
Total	2	3	3	13	11	33	149	116	316	272	84	2	1004
Mol wt by amino acid anal.	278	305	303	1256	941	3076	13,836	10,865	28,693	25,218	8080	245	93,096
Mol wt by agarose chromatography	278[b]	305[b]	303[b]	1256[b]	941[b]	3100	31,600	11,000	30,000	26,000	7800	245[b]	94,828

[a] Residues per peptide to the nearest whole number. Actual values are given where fewer than ten residues are found. [b] Molecular weight determined by amino acid analysis only. [c] Peptides isolated from bovine articular cartilage collagen are identical in size and amino acid composition with those presented here from bovine nasal cartilage collagen. Peptides from articular cartilage collagen, however, generally have slightly lower amounts of hydroxylysine and larger amounts of lysine than those derived from nasal cartilage collagen.

TABLE II: Amino Acid Composition[a] of CNBr Peptides from α1(II) of Human Articular Cartilage Collagen.

Amino Acid	1	2	3	6	8	(9,7)	10	11	12	15	Total CNBr Peptides
3-Hydroxyproline	0	0	0	0	0	2 (2.0)	0	0	0	0	2
4-Hydroxyproline	0	0	0	4 (4.0)	16	10	28	29	10	0	97

Aspartic acid	0	0	0	1 (1.0)	5 (5.1)	6 (5.8)	15	10	4 (4.1)	0	41	
Threonine	0	0	0	0	3 (3.0)	2 (2.1)	7 (7.1)	7 (7.1)	1 (1.0)	0	20	
Serine	0	0	0	1 (1.0)	3 (2.9)	5 (4.9)	8 (8.1)	5 (5.1)	3 (2.9)	0	26	
Glutamic acid	1 (1.0)	0	0	4 (4.2)	16	8 (8.1)	29	24	6 (6.1)	0	88	
Proline	0	0	1 (1.0)	6 (5.9)	16	19	38	32	8 (8.1)	0	119	
Glycine	0	1 (1.0)	1 (1.0)	11	50	38	107	90	28	0	326	
Alanine	0	0	0	2 (2.1)	14	7 (7.2)	39	29	6 (6.1)	1 (1.0)	98	
Valine	0	1 (0.8)	0	1 (1.0)	2 (2.1)	1 (1.0)	7 (6.8)	4 (4.0)	2 (2.0)	0	18	
Isoleucine	0	0	0	0	0	3 (2.9)	2 (2.2)	4 (3.8)	0	0	9	
Leucine	0	0	0	0	8 (7.9)	3 (3.1)	6 (5.9)	6 (6.1)	2 (2.0)	0	25	
Tyrosine	0	0	0	0	0	0	0	0	1 (1.1)	0	1	
Phenylalanine	0	0	0	1 (1.0)	3 (3.1)	1 (1.1)	3 (2.8)	4 (4.0)	1 (1.0)	0	13	
Hydroxylysine	0	0	0	0	2 (1.9)	2 (1.8)	3 (3.2)	4 (4.1)	3 (2.8)	0	14	
Lysine	0	0	0	0	4 (3.9)	1 (1.2)	8 (8.0)	6 (6.0)	3 (2.8)	0	22	
Histidine	0	0	0	0	0	1 (1.0)	0	0	1 (1.0)	0	2	
Arginine	0	0	0	1 (1.0)	6 (6.2)	6 (6.2)	15	17	4 (3.9)	1 (1.0)	50	
Homoserine	1 (1.0)	1 (0.9)	1 (0.9)	1 (1.0)	1 (0.9)	1 (1.0)	1 (1.0)	1 (0.9)	1 (1.0)	0	9	
Total	2	3	3	33	149	116	316	272	84	2	980	
Mol wt by amino acid anal.	278	305	303	3076	13,844	10,851	28,604	25,248	8037	245	90,791	
Mol wt by agarose chromatography	278[b]	305[b]	303[b]	3150	13,900	11,200	29,800	26,000	8000	245[b]	93,181	

[a] Residues per peptide to the nearest whole number. Actual values are given where fewer than ten residues are found. [b] Molecular weight determined by amino acid analysis only.

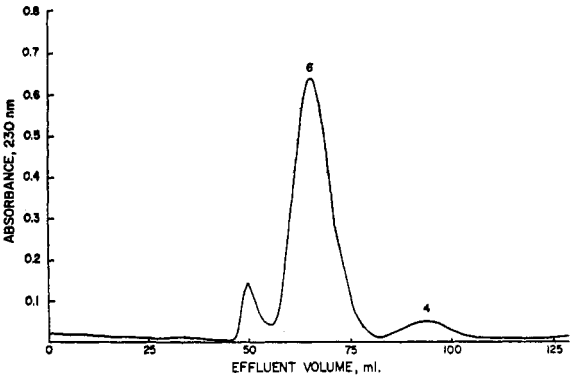

FIGURE 4: Bio-Gel P-6 rechromatography of the CM-cellulose eluent containing peptides 6 and 4 from bovine nasal cartilage collagen (effluent volume, 100–160 ml, Figure 3). The amount of peptide 4 recovered in this instance approximates 10% of the yield of peptide 6.

cross-linking in cartilage collagen (Miller, 1971c). Thus, the low recovery of peptide 4 and the absence of peptide 14 in CNBr digests of bovine nasal cartilage collagen may result from the association of these peptides with other sequences in the cartilage collagen through cross-linking.

A chromatogram illustrating the resolution of the larger CNBr peptides derived from human articular cartilage collagen is presented in Figure 6. The general features of the chromatogram are quite similar to those observed when CNBr peptides from either bovine nasal cartilage collagen (above) or chick cartilage collagen (Miller, 1972) are chromatographed under identical conditions. In human articular cartilage collagen, however, peptide 4 as well as peptide 14 were not present in the CNBr cleavage products. Moreover, as may be discerned by comparing Figure 6 with Figure 3, peptide 5 is also missing in the CNBr cleavage products of human articular cartilage collagen, suggesting that human α1(II) contains one less methionyl residue than bovine α1(II). Peptide 5 is an 11 amino acid sequence situated between the sequences represented by peptides 10 and 9 in chick α1(II) (Miller *et al.*, 1973) or between peptides 10 and (9,7) in bovine α1(II). The data presented in Table II for peptide (9,7) in human α1(II) indicates that the latter peptide does not contain an additional sequence belonging to peptide 5. Thus, we propose that the methionyl residue joining peptides 10 and 5 in chick and bovine α1(II) is not present in human α1(II).

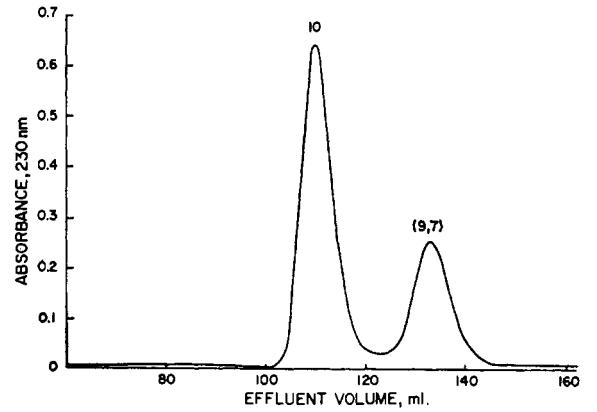

FIGURE 5: Agarose molecular sieve elution pattern illustrating the resolution of peptides 10 and (9,7) from bovine nasal cartilage collagen. Identical elution patterns were observed when these peptides from bovine or human articular cartilage collagen were chromatographed in this system.

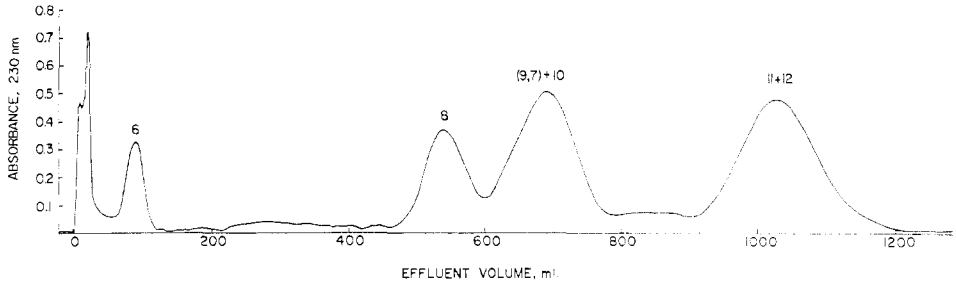

FIGURE 6: CM-cellulose chromatogram illustrating the resolution of the larger CNBr peptides from human articular cartilage collagen. The conditions of chromatography have been described (Miller, 1972).

Due to the large size of peptide 10, however, this possibility could not be definitively established on the basis of amino acid analyses alone.

Amino Acid Composition of the CNBr Peptides. Amino acid analyses of the peptides derived from bovine nasal cartilage collagen and human articular cartilage collagen are presented in Tables I and II, respectively. Molecular weights of the larger peptides were determined by molecular sieve chromatography on a calibrated column of agarose beads and in each case the results agree well with molecular weights determined by amino acid analysis. The total number of residues of each amino acid detected in the 12 CNBr peptides isolated from bovine nasal cartilage collagen and the 10 CNBr peptides from human articular cartilage collagen are also listed. These values must be considered as conservative, however, since the 19 amino acid sequence corresponding to peptide 14 was not observed in either the bovine nasal or human articular cartilage collagens. In addition, the short sequences represented by peptides 4 and 5 are not included in the data for human articular cartilage collagen. Nevertheless, in view of the virtual impossibility of obtaining intact α1(II) chains from the tissues under investigation, the data allow a reasonably accurate estimate of the amino acid composition of bovine and human α1(II). Indeed, these data indicate that relative to α1(I) of these species (Rauterberg and Kühn, 1971; Click and Bornstein, 1970; Epstein *et al.*, 1971) bovine and human α1(II) exhibit the same general compositional differences as previously observed between chick α1(II) and α1(I) chains (Miller, 1971a; Trelstad *et al.*, 1970). These differences include a relatively high content of glutamic acid and hydroxylysine and a diminished content of alanine in α1(II).

Discussion

The present study has demonstrated the feasibility of characterizing cartilage collagen at the level of the peptides released during CNBr cleavage of partially purified insoluble cartilage collagen preparations. Our data indicate that over 95% of the collagen in three difference cartilages may be recovered as soluble CNBr peptides and that the latter are readily isolated by a combination of ion-exchange and molecular sieve chromatography. In addition, in the course of these experiments, peptides originating either from α1(I) or α2 chains of bovine or human collagen were not detected in the CNBr cleavage products, indicating that all of the collagen in the tissues under investigation may be characterized as having the chain composition $[\alpha 1(II)]_3$.

The peptides derived from bovine cartilage collagen are quite similar to those from human cartilage collagen. For the smaller peptides (1, 2, 3, 6, and 15) the analyses are sufficiently precise to conclude that no differences in composition and presumably sequence occur. With respect to the larger peptides such as 8, (9,7), 10 (probably (10,5) in human cartilage collagen), 11, and 12, one can detect minor differences in hydroxylation of prolyl and lysyl residues as well as amino acid composition. Although further work will be required to establish the significance of the latter differences, it is likely that they are real and reflect species differences at the genetic level.

It is of interest to note that the amino acid compositions listed in Table II for peptides 8 and 12 from human articular cartilage collagen are, within experimental error, the same as those previously reported for their homologs from human epiphyseal cartilage (Miller *et al.*, 1971).

All of the peptides isolated in the present study are clearly recognizable as homologs of the CNBr peptides previously characterized from chick cartilage collagen (Miller, 1971b, 1972). Peptides 1 and 15, representing the NH_2- and COOH-terminal sequences of the α1(II) chain (Miller *et al.*, 1973), are identical in chick, bovine, and human cartilage collagens. The same may be indicated for peptides 2 and 3 which were not resolved in studies on chick cartilage collagen (Miller, 1971b) but which have the same total amino acid composition as the two tripeptides isolated in the present study. Peptide 4 from bovine cartilage collagen is likewise identical in composition and presumably sequence with peptide 4 from chick cartilage collagen. Peptide 5 from bovine cartilage collagen contains the same number of amino acids as peptide 5 from chick cartilage collagen although the compositional data indicate an alanine–serine substitution in the bovine sequence relative to the chick sequence. As indicated above, peptide (9,7) from bovine and human cartilage collagen accounts for the sequences represented by peptides 9 plus 7 in chick cartilage collagen. In this regard, it is of interest to note that in chick cartilage collagen peptides 9 and 7 each contain one 3-hydroxyprolyl residue and peptide 9 contains a single histidyl residue. In mammalian cartilage collagen, peptide (9,7) contains two 3-hydroxyprolyl residues and one histidyl residue. Similar comparisons can be made between peptides 10, 11, and 12 from bovine and human cartilage collagen and chick cartilage collagen. For instance, peptide 12 from the cartilage collagen of each species contains 84 amino acid residues and in each case the peptide contains one residue of tyrosine and one residue of histidine.

It should be further noted that peptide 6 from bovine and human cartilage collagen contains 33 amino acid residues whereas previous studies on peptide 6 from chick cartilage collagen indicated the presence of 36 amino acids in this sequence (Miller, 1971b). It is now known that the latter value is incorrect and that peptide 6 in all three species contains 33 amino acids. Moreover, peptide 6 occupies the same general region in α1(II) as does α1-CB2 in α1(I) (Miller

et al., 1973). Furthermore, sequence studies on peptide 6 (Butler, W. T., and Miller, E. J., submitted for publication) indicate that its primary structure corresponds quite closely to that of residues 4–36 in α1-CB2 (Bornstein, 1967). These data strongly suggest that one of the tripeptides (peptide 2 or 3) comprises the α1(II) sequence corresponding to residues 1–3 in α1(I)-CB2. The most likely choice is peptide 3 (a glycine-proline-methionine sequence) corresponding to the sequence glycine-proline-serine in α1(I)-CB2.

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Isolation of the Nonhistone Proteins of Rat Walker Carcinoma 256. Their Association with Tumor Angiogenesis†

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ABSTRACT: A procedure is described to isolate nonhistone proteins of malignant cell nuclei for the purpose of testing certain of its biologic activities. Walker rat carcinoma 256 cells were disrupted by N_2 cavitation, the nuclei isolated by differential centrifugation, the chromatin was purified, and the DNA separated from chromatin protein by chromatography on Bio-Gel A 5m in the presence of 4 M guanidine-HCl. Further separation of histone and nonhistone proteins was achieved with CM-Sephadex C-50, eluting alkylated nonhistones with 0.4 M guanidine-HCl in 0.01 M phosphate buffer and subsequent elution of histones in 2 M guanidine-HCl in 0.1 M acetic acid. Nonhistone and histone proteins were obtained in good yield and satisfactory purity. The histone and nonhistone proteins were characterized by amino acid analysis, macromolecular composition, and gel electrophoresis. A fraction containing tumor nonhistone proteins was mitogenic to endothelial cells, and, when implanted into the rabbit cornea, caused proliferation of vascular endothelium and new blood vessel formation. Tumor angiogenesis factor was thus demonstrated to be associated with a fraction also containing nonhistone proteins of malignant cell nuclei. Histone fractions had no activity in this bioassay.

The deoxyribonucleic acid in eukaryotic organisms is present in the cell nucleus not as free DNA but in the form of chromatin, a chemically complex material composed of nucleic acids, relatively constant amounts of histone, and a variable amount of nonhistone proteins. The nonhistone proteins, unlike histones, vary both qualitatively and quantitatively with developmental stages (Marushige and Ozaki, 1967; Hill *et al.*, 1971) and from tissue to tissue of a given organism (Dingman and Sporn, 1964; Elgin and Bonner, 1970; Chytil and Spelsberg, 1972). *In vitro* template assay of isolated chromatin has established a direct relationship between the amount of nonhistones present and the template activity of isolated chromatin (Bonner *et al.*, 1968). In general,

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