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Properties of a Collagen Molecule Containing Three Identical Components Extracted from Bovine Articular Cartilage*

Elsa Strawich† and Marcel E. Nimni‡

ABSTRACT: Incubation of bovine articular cartilage with papain at 4° followed by several washes with 0.15 M NaCl removes most of the hexosamines, uronic acid, sialic acid, and non-collagen-bound hexoses. Subsequent extraction with 0.45 M NaCl (pH 7.0) causes 18–20% of the collagen to appear in solution. This collagen is still associated with significant amounts of glycosaminoglycans and can be considerably purified by successive reprecipitations. It behaves like native collagen as judged by its viscosity, optical rotation, and melting profile and when denatured gives rise to one single component, with electrophoretic and chromatographic properties similar to the α1 chain from calf skin collagen. It differs from this type of collagen primarily by its high content of hydroxylysine (28 residues/1000) and glycosidically bound carbohydrate (8–9 glucose and 13 galactose residues per α chain). The fact that no other type of collagen chain was observed indicates that articular cartilage is composed entirely of a triple stranded molecule of the (α1-Type II)$_3$ conformation, similar to that first observed by Miller and Matukas ((1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1264) in the sternal cartilage of lathyritic chicks. Evidence is also presented which suggests that collagen in articular cartilage may have lesser amounts of covalent cross-links than bone or skin, and may be stabilized to a great extent by interactions with the proteoglycan components of the ground substance.

Articular cartilage is a unique tissue in both structure and function. It is almost completely avascular, with a low cell density, composed primarily by extracellular substances such as collagen, proteoglycans, and glycoproteins.

So far, its principal components, collagen and proteoglycans, have been difficult to study in their native form due to problems involved in their isolation. The conventional procedures used to extract collagen using hypertonic salt solutions or dilute organic acids do not yield any material, and more drastic conditions yield degraded or denatured products. Because of this problem most studies have involved other types of cartilage, even though articular cartilage is of primary significance because of its involvement in the pathogenesis of joint disease. Sadjera and Hascall (1969) obtained several preparations of proteoglycans from bovine nasal cartilage by disruptive and dissociative techniques and Rosenberg *et al.* (1970) compared the yield and composition of the fractions isolated by similar methods. However, none of these procedures enhanced a subsequent extractability of collagen from articular cartilage. Miller *et al.* (1969) were only able to extract about 1% of the total collagen present in normal human articular cartilage using 5 M guanidine hydrochloride. Subsequently, Miller and Matukas (1969) and Miller (1971) described a new type of α1 chain in the sternal cartilage of lathyritic chicks. These investigators suggested that cartilage contained two types of collagen: one similar to that present in skin and bone, and one unique to this tissue containing only α1 chains. The two species of collagen present in lathyritic chick xiphoid cartilage were recently separated by Trelstad *et al.* (1970) after introducing intramolecular cross-links with formaldehyde followed by chromatography on CM-cellulose.

The present experiments describe the isolation and the properties of a unique type of collagen from articular cartilage containing three identical α1 chains.

Materials and Methods

Extraction Procedure. Bovine articular cartilage from long bones was obtained from a slaughterhouse. Cartilage was removed, freed from adhering tissues, rinsed with saline, and frozen. Sliced samples (5 g) were digested without shaking with 30 ml of 0.1% papain (Calbiochem) in 0.02 M phosphate buffer containing 2×10^{-2} M cysteine and 3×10^{-3} M EDTA at 4° for 48 hr. The suspension was centrifuged at 30,000*g* for 30 min. The sediment was resuspended in 0.15 M NaCl (pH 7.0) and the enzyme inactivated with 1×10^{-3} M iodoacetic acid, centrifuged, and washed twice with a similar solution. The insoluble material was dispersed in 0.45 M NaCl (pH 7.0)

* From the Rheumatic Disease and Immunology Section, Departments of Medicine and Biochemistry, School of Medicine, University of Southern California, Los Angeles, California 90033. *Received May 10, 1971.* This research was supported by grants from the National Institutes of Health (AM 10358 and DE-02471) and from the Arthritis Foundation.

† Visiting Research Fellow from the Centro de Investigaciones Medicas Albert Einstein, Buenos Aires, Argentina.
‡ To whom to address correspondence.

with a Virtis-45 homogenizer at high speed for 2 min, and then shaken for 48 hr. After centrifuging at 30,000*g* the residue was again extracted with 0.45 M NaCl. The combined supernatants were dialyzed against water and 0.02 M disodium phosphate. The precipitated material was dissolved in 0.5 M acetic acid and salted out twice with 5% sodium chloride. The last precipitate was dissolved in 0.45 M NaCl (pH 7.0) and salted out with 20% NaCl. The precipitate was suspended in 0.45 M NaCl (pH 7.0), dialyzed against the same buffer, and centrifuged at 100,000*g* for 90 min, and the supernatant used as a source of purified collagen. All these steps were performed at 4° and collagen estimations were carried out by determining hydroxyproline content by an automated version of the Stegemann procedure (1967). The moisture content of cartilage was obtained from samples rinsed with acetone and dried at 110° to constant weight.

Extraction of Collagen and Hexosamines from Cartilage by Various Other Reagents and Enzymes. Finely cut articular cartilage was extracted with various concentrations of salt solutions, denaturing agents, and enzymes. Chemical extractions were aided by constant shaking while enzymatic extractions were not. The 30,000*g* supernatants were analyzed for hydroxyproline and hexosamines and the values expressed as a per cent of the total material present in the original specimen.

Hexose Analysis. Total hexoses were measured by the anthrone method (Roe, 1955). A solution containing galactose–glucose (1:1, v/v) was used as a standard. Samples were hydrolyzed in 2 N HCl at 110° for 2 hr as described by Butler and Cunningham (1965). The hexoses were separated by chromatography on a Dowex 50X-2 column (200–400 mesh, H + form) by eluting with water. The anthrone-positive material was pooled, lyophilized, dissolved in water, and spotted along with the standards on Whatman No. 1 paper. The chromatograms were developed with 1-butanol–pyridine–water (6:4:3, v/v). After locating the standards with aniline–phthalate the chromatograms at corresponding places were cut out, eluted, and tested by the anthrone method.

Hexosamine Estimation. D-Glucosamine hydrochloride (Calbiochem) was used as a standard. Samples containing 1–2 mg of collagen/ml were hydrolyzed in 4 N HCl at 100° for 8 hr and were analyzed using Boas (1953) modification of the Elson and Morgan method.

Uronic Acid Analysis. Uronic acid was estimated by the carbazole method modified by Bitter and Muir (1962). The samples were previously hydrolyzed in 2 N HCl at 100° for 2 hr. Glucuronic acid (Calbiochem) was used as a standard.

Sialic Acid. It was evaluated by the thiobarbituric assay of Warren (1959) after the samples were hydrolyzed in 0.1 N H_2SO_4 at 80° for 1 hr. The sialic acid used as a standard was purchased from Sigma Chemical Co.

Viscosity Studies. Samples were dissolved in 0.05 M acetic acid and centrifuged at 100,000*g* for 90 min. Intrinsic viscosity values were obtained at 15° using an Ostwald or a Zimm–Crothers (1962) low-shear viscometer (Beckman Instruments, Inc.).

For determining the melting temperature collagen was dissolved in 0.05 M acetic acid and equilibrated at each temperature for 10 min.

Optical Rotation. It was estimated with a Zeiss spectropolarimeter using a 50-mm light-path cell at 365 mμ. Readings were taken at 20°.

Amino Acid Analysis. Samples of whole cartilage, a trichloroacetic extract of cartilage (Fitch *et al.*, 1955), a water extract, as well as the purified collagen extract and the material chromatographed from CM-cellulose were analyzed. Extraction with 0.3 M trichloroacetic acid was carried out at 90° for 30 min and the extract dialyzed against water and lyophilized. The water extract was obtained from cartilage samples heated under reflux for 5 hr. Hydrolysis was performed in 6 N HCl at 108° for 24 hr in sealed tubes under nitrogen. The analysis was carried out in a Jeol automatic amino acid analyzer.

Calf Skin Collagen. Skin samples from fetal calves were cleaned and extracted with 0.5 M acetic acid. The crude extract was purified by repeated salting out with 5% NaCl at an acid pH and various precipitations by dialysis against 0.02 M Na_2HPO_4.

Carboxymethylcellulose Chromatography. Sixty to one-hundred milligrams of each type of collagen was chromatographed in the denatured form on a 2.8 × 20 cm CM-cellulose column (Whatman microgranular CM-32, capacity 1.0 mequiv/g) equilibrated with 0.05 M sodium acetate buffer (pH 4.8) at 40°. The elution was carried out using a linear gradient of NaCl between 0 and 0.1 M (Piez *et al.*, 1963). A flow rate of 150 ml/hr was maintained using an LKB ReCyChrom peristaltic pump. The effluent was monitored at 230 mμ with a Uvicon-540 ultraviolet absorption meter and the absorbancy recorded on a Uvicon TU-200 recorder. Fractions of 10 ml were collected and the material under the peaks was desalted and lyophilized.

Acrylamide Disc Electrophoresis. The method of Stark and Kuhn (1968) was used. The upper gel consisted of 2.5% acrylamide and the lower gel of 7.5% acrylamide and included 12% urea. The samples were saturated with urea. The electrophoresis was carried out for 4 hr using a current of 4 mA/tube.

Incubation of Other Tissues and Soluble Collagen with Papain. The object of these experiments was to observe how papain under the conditions used to remove the proteoglycans from cartilage would affect collagen in solution and in the native fibrillar form.

Soluble collagen from rat or calf skin (2.0–2.5 mg/ml) was incubated with 0.1% papain at 4°, in the presence of cysteine and EDTA for periods of 24, 48, and 72 hr. Rat and calf skin were used as sources of fibrous collagen. After freeing from adhering tissue they were washed with 0.15 M NaCl, cut into small pieces, and incubated with the papain solution for the same lengths of time. After inactivating the papain, acid-soluble collagen was extracted from these tissues with 0.5 M acetic acid or 0.45 M NaCl (pH 7.0) at 4°. Collagen was purified as previously described, and analyzed for the presence of α and β components by acrylamide disc electrophoresis or CM-cellulose chromatography.

Periodate Oxidation. Collagen samples were treated with 0.3 M sodium periodate at room temperature for 5 hr as described by Aronson *et al.* (1967). Excess of periodate was removed using an ion-exchange resin (AG 1-X8) followed by dialysis. The samples were hydrolyzed with 6 N HCl and analyzed for hydroxylysine content on the amino acid analyzer.

Aldehyde Estimation. Samples were dissolved in 0.1 M glycine–HCl buffer (pH 4.0) and gelatinized, and the aldehydes estimated by the spectrophotometric method of Paz *et al.* (1965).

Results

Table I shows the composition of bovine articular cartilage from long bones. The values are expressed on a per cent basis and refer to the dry weight of the tissue. Articular cartilage contains 74–75% water. The values for collagen are very close

TABLE I: Carbohydrate and Collagen Content of Bovine Articular Cartilage.

	% of Dry Tissue
Collagen	67.2
Total hexosamines	5.6
Uronic acid	4.5
Sialic acid	0.6
Hexoses	10.0

to those previously reported by Campo and Tourtellotte (1967) for young articular cartilage.

The amounts of collagen and hexosamines extractable from cartilage over a 48-hr period under a variety of experimental conditions are shown in Table II. Distilled water extracted significant amounts of collagen, hexosamines, uronic acid, and sialic acid. The main disadvantage associated with the use of this solvent was the difficulty encountered in attempting to isolate significant amounts of native collagen from this mixture. Neutral salt solutions yielded smaller amounts of soluble collagen, and the same difficulties became apparent during high-speed centrifugation since the collagen coprecipitated with the glycosaminoglycans. Extraction with acetic acid at 60° yielded degraded products and so did most of the enzyme extractions, particularly if incubations were carried out at 37°. On the other hand, none of the chemical procedures affected the residual cartilage so as to enable collagen to become more readily extractable. Pretreatment with papain at 4° followed by an increase in the ionic strength of the extracting solution seemed to provide the best experimental approach in this connection. The cartilage components extracted by papain digestion are shown in Table III. Following 48-hr incubation at 4°, large amounts of glycosaminoglycans and only little collagen were released into the media. After inactivating the enzyme, washing with 0.15 M NaCl caused a further release of glycosaminoglycans from the cartilage with only traces of hydroxyproline. Once most of the proteoglycans were removed, large amounts of collagen (18–22% of the total) could be extracted with 0.45 M NaCl (pH 7.0). There were significant amounts of carbohydrates associated with this fraction which could be partially removed by repeated NaCl precipitations.

The molar concentrations of hexosamines, uronic acid, sialic acid, and hexoses present in the crude 0.45 M NaCl extract, before and after precipitations by dialysis against 0.02 M Na_2HPO_4, salting out, and high speed centrifugation, are shown in Table IV. Approximately 5% of the total collagen present in the articular cartilage was recovered in this final purified fraction after high-speed centrifugation. The major peak which appears during chromatography on CM-cellulose is also included in this table. Figure 1 shows the elution patterns of collagen from bovine articular cartilage and skin collagen. The hexoses which remain tightly bound to the

TABLE II: Percentage of the Total Collagen and Hexosamines Present in Articular Cartilage Solubilized under Various Experimental Conditions.[a]

	Collagen	Hexosamines
Distilled water, 4°	5.1	29.9
0.15 M NaCl, 37°	0.1	7.1
0.15 M NaCl, 60°	1.3	25.0
0.45 M NaCl, 37°	0.2	2.2
0.45 M NaCl, 60°	1.9	16.0
2 M $MgCl_2$, 4°	2.2	13.6
2 M $CaCl_2$, 4°	1.1	23.2
0.5 M Acetic acid, 60°	15.0	14.0
5.0 M Guanidine hydrochloride, 37°	0.8	21.0
5.0 M Guanidine hydrochloride, 60°	1.0	30.4
6.0 M Urea, pH 8, 37°	1.0	2.0
Pronase, 20°	4.0	36.0
Papain, 4°	0.4	71.0
Papain, 37°	53.0	63.0
α-Amylase, 4°	2.0	84.0
Chymopapain, 37°	0.7	52.0
Pepsin, pH 2.0	6.1	25.5
10^{-2} M Cysteine + 10^{-3} M EDTA	0.3	

[a] Unless specified to the contrary, reactions were carried out at pH 7.0 over a 48-hr period.

TABLE III: Percentage of the Total Carbohydrates and Collagen Present in Cartilage Which Became Solubilized Following Papain Treatment at 4° Followed by 0.45 M NaCl (Crude Collagen Extract) after Inactivation of the Enzyme.[a]

	Collagen	Hexosamines	Uronic Acid	Sialic Acid	Hexoses
Papain					
Supernatant	0.31	73.0	84.7	60.0	45.0
First wash	0.20	7.4	4.5	20.0	7.6
Second wash	0.20	1.4	1.3		6.0
Third wash	0.01	0.8	0.3		2.7
Crude extract (0.45 M NaCl)	18.9	7.8	2.3	7.0	17.0

[a] All values are expressed as a per cent of the total amounts of the individual components present in the original cartilage.

TABLE IV: Carbohydrate Content of Various Collagen Fractions during the Purification Procedure (Expressed as Moles of Carbohydrate per Mole of Collagen).[a]

	Component			
Collagen Fraction	Hexosamines	Hexoses	Uronic Acid	Sialic Acid
Crude 0.45 M NaCl extract	56	86	13.3	3.9
Second precipitation with 5% NaCl from 0.5 M acetic acid	30	66	4.1	2.3
Precipitated with 20% NaCl, pH 7.0	13.4	66	4.0	2.0
100,000*g* Supernatant	6.5	64	3.0	1.3
CM-cellulose peak α1 (II)	2.0	64	*b*	*b*

[a] Molecular weight of collagen = 280,000. [b] Traces.

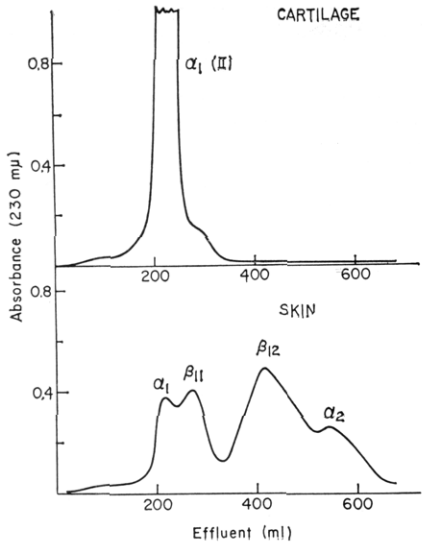

FIGURE 1: Chromatography of denatured collagen subunits on CM-cellulose. Upper graph corresponds to the purified collagen fraction extracted from cartilage; lower graph to collagen extracted from calf skin.

purified collagen were separated by paper chromatography and revealed a glucose:galactose ratio of 1.6.

Only trace amounts of β components were detected in the crude or purified extracts of cartilage collagen as well as in the collagen extracted in lesser amounts by nondegradative procedures, such as 1 M $MgCl_2$, 1 M NaCl, or water. All of these fractions showed one single component which migrated like $\alpha 1$ from bovine skin (Figure 2B).

Nevertheless, because of our concern that papain could be cleaving the molecule near the region where the intramolecular cross-link is located, additional experiments were planned to indirectly test this possibility. For this purpose, calf or rat skins, cut into small pieces, were incubated with papain for either 48 or 72 hr, the enzyme inactivated, and then the collagen extracted from these tissues with 0.5 M acetic acid or with 0.45 M NaCl (pH 7.0). The collagen solubilized under these conditions showed no change in the ratio of α to β components when compared to similar material extracted in the absence of

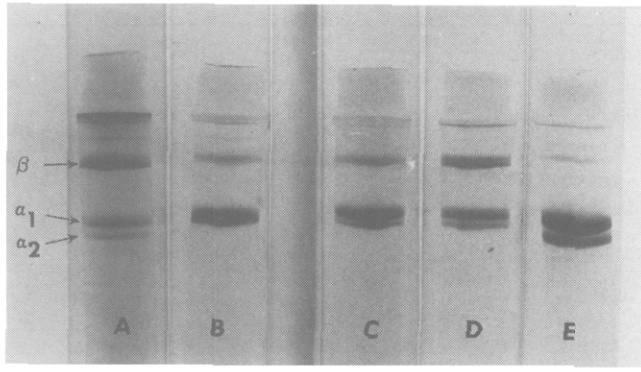

FIGURE 2: Separation of collagen subunits by acrylamide disc electrophoresis. (A) Acid-soluble collagen from calf skin; (B) collagen fraction extracted from articular cartilage previously incubated with papain for 48 hr at 4° to remove the proteoglycans; (C) collagen fraction extracted from rat skin by 0.45 M NaCl (pH 7.0) at 4°; (D) collagen fraction extracted by 0.45 M NaCl from rat skin previously incubated with papain under the same conditions as cartilage; (E) soluble collagen incubated in solution with papain at 4° for 48 hr.

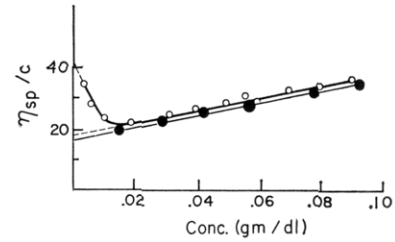

FIGURE 3: Viscosity measurements performed on the purified collagen fraction extracted from cartilage. The open circles correspond to measurements performed using the Zimm–Crothers low-shear viscometer; closed circles to results obtained with an Ostwald viscometer.

papain treatment (Figure 2D). On the other hand, if acetic acid or neutral salt soluble collagen from calf and rat skin containing significant amounts of β components were incubated in a media containing papain the results were quite different. After 24-hr incubation at 4° a significant proportion of the intramolecular cross-links were cleaved. By 48 hr there was an almost completete conversion of β components into α-like components (Figure 2E), and after 72 hr some degradation of the α chains into smaller fragments could be seen (results not shown).

These experiments clearly show how the N terminus of collagen becomes resistant to papain when the molecules are organized into fibers, whereas when they are free in solution they are susceptible to cleavage in the nonhelical region.

The collagen fraction extracted from cartilage by 0.45 M NaCl behaved in all respects like native soluble collagen. The changes in relative viscosity as a function of concentration measured with Zimm–Crothers and Ostwald viscometers are shown in Figure 3. Cartilage collagen yields a slightly higher value than neutral salt soluble collagen from rat or calf skin, and resembles acid-soluble collagen from skin. Linear extrapolation to zero at low-shear rates (or at high rates of shear using the Ostwald viscometer) gave values ranging between 17 and 18 dl per g (neutral salt soluble from rat skin = 12 dl/g; acid-soluble collagen = 19–22 dl/g). With the Zimm–Crothers low-shear viscometer the reduced viscosity begins to increase at collagen concentrations below 0.01 g/ml. This is a common feature for all types of collagen.

The denaturation of articular cartilage collagen dissolved in dilute acetic acid occurred over a temperature range which resembles that for most mammalian collagens. The T_m for the purified collagen was 39° (Figure 4).

Specific optical rotation measured at 365 mμ was found to be −1040. This value dropped to −484 after heat denatura-

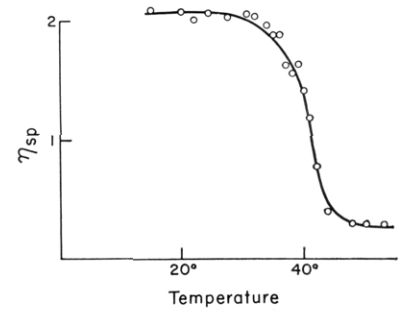

FIGURE 4: Heat denaturation curve of cartilage collagen dissolved in 0.1 M acetic acid.

TABLE V: Amino Acid Composition of Bovine Cartilage and Various Extracted Fractions as Well as the α1(I) Chain from Bovine Skin.

	Articular Cartilage					
	Whole Tissue	Trichloroacetic Acid Extract	Hot Water Extract	Purified Collagen Fraction	α1(II)	Skin α1(I)
Hydroxylysine	24	28	29	29	28	8
Lysine	19	19	19	15	15	26
Histidine	9	6	4	5	6	5
Arginine	53	47	48	48	46	50
Hydroxyproline	82	85	83	95	95	84
Aspartic acid	57	54	51	51	49	47
Threonine	31	24	22	19	21	22
Serine	36	30	31	28	27	40
Glutamic acid	100	87	86	82	80	80
Proline	106	109	103	115	114	135
Glycine	295	319	325	330	329	329
Alanine	69	103	100	98	103	108
Valine	22	17	24	19	20	12
Methionine	13	11	12	9	10	7
Isoleucine	16	14	12	13	12	11
Leucine	37	26	29	27	26	22
Tyrosine	6	2	3	2	2	2
Phenylalanine	21	20	18	14	15	14

tion, these ranges being similar to those for acid-soluble collagens from rat and calf skin.

The amino acid analysis of whole cartilage, as well as that of the various extracts and the purified fractions, can be seen in Table V. Included in this table is α1(I) from bovine skin. Since the protein of articular cartilage consists primarily of collagen the amino acid values of all the fractions studied do not differ very significantly among themselves. The collagenous material purified by dialysis and salt precipitations did not differ from that which eluted as a major peak during CM-cellulose chromatography. The major differences between the cartilage collagen and the corresponding α1 chain from bovine skin can be traced to the presence of large amounts of hydroxylysine with a concomitant decrease in lysine. In addition, the α1(II) collagen extracted from bovine articular cartilage contained less serine and more valine and leucine than the comparable chain isolated from the skin of the same species. Table VI summarizes the most significant properties that characterize the α1 (type II) collagen subunit present in bovine articular cartilage.

Discussion

Although the collagenous nature of the fibers in articular cartilage has never been in doubt, their properties seem to be different from other tissues. The fibers are thinner, more closely associated with the polysaccharide matrix, and oriented in an arcade fashion which makes observation of long stretches of fibers very difficult.

Proteolytic enzymes which cleave the protein backbone of the protoglycans have been of great help in attempting to elucidate the chemistry of the ground substance. In particular, papain has shown a marked specificity for depolymerizing the proteolgycan complexes of cartilage. Intravenous injection of crude papain causes a softening and eventual collapse of the normally erect ear of rabbits (Thomas, 1956). This is accompanied by a marked decrease in the basophillic and metachromatic staining and of the chondromucoprotein content of cartilage (Spicer and Bryant, 1957; Tsaltas, 1958). The collagen matrix does not seem to be affected and these changes are reversed within a few days. Purified papain will cause a rapid disintegration of bovine articular cartilage at physiological pH and temperature (Schneider and Goldstein, 1967). A similar effect of papain had been previously demonstrated using chondroprotein from pig laryngeal cartilage as a substrate (Muir, 1958). Due to the temperatures used in most experiments involving papain (37–60) collagen becomes rapidly degraded. Recently, DiFerrante and Neri (1971) showed that crude bacterial α-amylase extracted significant amounts of

TABLE VI: Fundamental Properties of the α1(II) Chain Present in Bovine Articular Cartilage.

	Residues/1000 Residues of Amino Acids
Hydroxylysine	28
Periodate-labile hydroxylysine	14
Galactose	14
Glucose	8–9
Hexosamines	2.0
Aldehyde content	0.2–0.3
Mobility on acrylamide gel elution from CM-cellulose sedimentation pattern	Similar to α1(I)

chondroitin sulfate from rabbit ear cartilage. This effect seemed to be due to the protolytic activity associated with the enzyme preparation which digested the protein backbone.

During the course of our experiments it became quite evident that the difficulties encountered in isolating collagen from cartilage were related to the presence of such large amounts of glycosaminoglycans in this tissue. After removal of significant amounts of these substances by papain at 4°, the collagen could be solubilized by increasing the ionic strength of the media. The residual glycosaminoglycans in this crude extract tend to coprecipitate with collagen during high-speed centrifugation and only after most of these are removed does collagen remain in solution.

The interactions of collagen with glycosaminoglycans have been a subject of intensive investigation. Using the technique of free solution electrophoresis Mathews (1965) detected a complex formation between collagen, hyaluronate, and chondroitin sulfate. The stability of these complexes appears to be mainly dependent on ionic interactions with contributions from molecular entanglements and excluded volume effects (Disalvo and Schubert, 1966; Laurent and Pietruszkiewicz, 1961; Fessler, 1960; Toole and Lowther, 1968). The dissociating effect of temperature suggests that hydrogen bonding may also play an important role in protein–polysaccharide interactions (Doyle *et al.*, 1968).

The purified collagen extracted from articular cartilage exhibits all the characteristics of native soluble collagen as judged by its high intrinsic viscosity, its optical rotation, and melting temperature profile. After denaturation it gives rise to one single component with the same electrophoretic mobility, elution pattern from CM-cellulose, and sedimentation velocity as the $\alpha 1$ chain from skin collagen. It contains 22 hexose molecules/1000 amino acid residues, out of which 8–9 are glucose and 13 galactose. All these galactose residues seem to be bound O glycosidically to hydroxylysine. After periodate oxidation the hydroxylysines with attached sugars were not degraded and could be recovered in the acid hydrolysate.

The amino acid composition of the $\alpha 1$ chain present in articular cartilage differs from the homologous chain present in skin, the fundamental difference being associated with a high hydroxylsine content. This increase in hydroxylation also becomes manifest when looking at the hydroproline values which increase at an expense of a decline in proline residues. There are lesser amounts of serine and more valine and leucine in the collagen isolated from cartilage. The amino acid analysis represents the average of many determinations; it should be emphasized that slight variations did occur from preparation to preparation, particularly in the degree of hydroxylation of lysine. The reason for such variability is not apparent but age and heterogeneity of the cartilage material are likely factors. Following the terminology proposed by Miller and Matukas (1969), we have called this type of collagen subunit $\alpha 1$ (type II) to distinguish it from that of skin and bone $\alpha 1$ (type I) or plain $\alpha 1$.

Collagen from various species and tissues has been shown to contain hydroxylysine-bound carbohydrates (Butler and Cunningham, 1965; Kefalides, 1969). In glomerular basement membrane which has a large amount of covalently bound carbohydrate, the attachment is in the form of 2-*O*-α-D-glucosyl-*O*-β-D-galactosylhydroxylysine (Spiro, 1969). It has been pointed out (Spiro, 1970) that collagens having the largest amounts of hydroxylysine-linked hexoses exhibit a lesser degree of morphological organization. It has also been suggested that in the good fiber forming collagens, such as those originating from dermis, the dissaccharide unit may be fitted into spaces or holes between molecules (Morgan *et al.*, 1970). The more glycosylated collagens will either have to attain a less ordered pattern or form thinner fibrils with the dissaccharide units on the surface. Another alternative is that these carbohydrate residues are restricting formation of covalent cross-links.

Although the collagen fractions extracted from cartilage seem to be deficient in intramolecular and intermolecular cross-links this observation becomes difficult to ascertain conclusively at this time. Alternative nonenzymatic extraction procedures, as well as the CNBr peptides derived from this collagen, are being investigated for this purpose. Nevertheless, it is pertinent that whereas soluble collagen from bovine or rat skin, with significant amounts of β components, can be cleaved near the region of the intramolecular cross-link to yield α-like subunits, fibrous collagen from the skin of these species is not attacked by papain at 4°. Incubation of skin with papain followed by extraction with acetic acid or 0.45 M NaCl yielded the same amount of intramolecularly cross-linked subunits as collagen from untreated tissues.

Using this experiment as an analogy to the cartilage extraction procedure, one can infer that the large amount of α chains present in this collagen may reflect ineffective cross-linking of this collagen. An opposing alternative could be that the cross-linking sites present in cartilage collagen fibers are more exposed or more sensitive to the action of papain than other polymeric collagens.

The fact that practically no β subunits could be detected in the water and salt-extracted fractions and that very low amounts of aldehydes are detectable in this collagen may also be a reflection of the sparcity of cross-links. Also, in this connection, it should be mentioned that Glimcher *et al.* (1969) found that the material extracted as gelatin by 9 M LiCl at 0° from chick embryo femors consisted primarily of α chains.

The presence of a single $\alpha 1$(II) component in bovine articular cartilage supports the suggestion made by Miller *et al.* (1971) that cartilage collagens represent a distinct species of closely homologous molecules with intrinsic structural features which make them more able to fulfill the mechanical requirements of such tissues.

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Oxidation of the Cysteine-Containing Histone F3. Detection of an Evolutionary Mutation in a Conservative Histone*

Sakol Panyim,† K. R. Sommer, and Roger Chalkley‡

ABSTRACT: All creatures we have examined, including plants, invertebrates, and vertebrates up to and including rodents, contain a single cysteine residue in their F3 histone. Mammals more highly evolved than rodents contain two such cysteine residues. The presence of the additional cysteine affords a more complex oxidation pattern and this has been documented. Thus, in contrast to those F3 molecules which contain a single cysteine residue and have a single dimer oxidation product, the F3 molecules with two cysteine residues can form an intramolecular disulfide bond (in solutions of high dielectric constant or at pH 8.0), a series of dimers with one or two disulfide bonds (in aqueous acetic acid), or a series of higher polymers (at high monomer concentrations). There is no evidence for histone disulfide-bond formation in interphase nuclei.

There are five major groups of histones in the eucaryote nucleus. These can be divided into three types on the basis of their chemistry and their resistance to evolutionary changes in primary structure as measured directly (DeLange *et al.*, 1969) or indirectly using electrophoresis (Panyim *et al.*, 1971), as follows: (1) the arginine-rich histones (F2a1 and F3) in which the primary structure is essentially conserved, (2) the moderately lysine-rich histones (F2b and F2a2) which show a somewhat more frequent change in primary structure during evolution, and (3) the lysine-rich histone (F1), the structure of which is almost species specific.

It is now well recognized that histones from diverse organisms invariably possess a single fraction which contains cysteine (Fambrough and Bonner, 1968; Panyim *et al.*, 1970). Gentle oxidation gives rise to a dimer with a characteristic electrophoretic mobility (Panyim and Chalkley, 1969). Such a dimer is observed as a single electrophoretic band on acrylamide gel electrophoresis. We have observed in this laboratory over the last few years that in contrast to most other species, calf histones give rise to two slower moving electrophoretic bands upon oxidation (Panyim and Chalkley, 1969) as well as developing a species capable of migrating even faster than the parent molecule. Since Bonner had suggested that calf (Fambrough and Bonner, 1968) and also human (Sadgopal and Bonner, 1970) histones contain two cysteine residues, these observations might be explained in terms of two forms of dimeric oxidation products as well as an intramolecular monomer oxidation product.

The following experiments were undertaken to try to establish the molecular nature of the various oxidation prod-

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† Present address: Department of Biochemistry, Mahidol University, Bangkok, Thailand.

‡ To whom correspondence should be addressed.