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Isolation of Two Distinct Collagens from Chick Cartilage*

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ABSTRACT: Two different molecular species of collagen have been isolated from neutral extracts of lathyritic chick xiphoid cartilage, supporting the suggestions and extending the observations of Miller and Matukas (1969). In the first extract the ratio of $\alpha 1$ to $\alpha 2$ is 4, whereas in later extracts it is as much as 30, indicating that an excess of $\alpha 1$ chains are present in cartilage and that the collagen in the later extracts is comprised only of $\alpha 1$ chains. Amino acid and carbohydrate analyses of this $\alpha 1$ chain ($\alpha 1$ type II) indicate it is different from the $\alpha 1$ chain ($\alpha 1$ type I) of skin and bone. In order to separate the two collagen molecules without dissociation into their component α chains, dilute solutions were treated with formaldehyde to introduce intramolecular cross-linkages. The cross-linked molecules were denatured and separated by chromatography on carboxymethylcellulose. The chain composition of the two molecules as determined by amino acid composition and chromatographic behavior is $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$. Over 90% of the extractable collagen from the lathyritic xiphoid cartilage is $[\alpha 1(II)]_3$. Electron micrographs of segment long spacings of cartilage collagen revealed a different and reproducible band pattern from that observed for chick skin collagen.

Changing staining and solubility characteristics of collagen during embryonic growth and wound repair, as well as differences in properties of this structural protein among the different tissues, had led us to search for the existence of different types of collagen, the changing distribution of which might be analogous to that of other proteins such as hemoglobin. However, it remained for Miller and Matukas (1969) to make the first definitive observation of two distinct $\alpha 1$ chains in cartilage differing in amino acid composition and suggesting the existence of two molecular species. In this paper we wish to add to their observations and to use cartilage as a model for further studies on other tissues.

Soluble collagen from a variety of vertebrate tissues yields three α chains of identical size (mol wt ~100,000) and their covalently cross-linked β components (mol wt ~200,000) when the collagen is denatured and chromatographed on carboxymethylcellulose. In most collagens so far examined two of the chains ($\alpha 1$) are identical with each other, but differ significantly from the other ($\alpha 2$) in amino acid composition and chromatographic behavior. The most notable exception to date of this pattern has been codfish skin collagen

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in which three distinct α chains have been found (Piez, 1965). Miller and Matukas (1969) have presented evidence that xiphoid cartilage collagen from the chicken contains two different α1 chains which they have called α1 type I and α1 type II which differ in chromatographic behavior, amino acid composition, and in at least one cyanogen bromide peptide, CB-2. They have suggested that cartilage collagen consists of two distinct populations of collagen molecules, α1(I)α1(I)α2 and α1(II)α1(II)α1(II).

Methods are not yet developed for separating two different collagen molecules in their native state. However, recent studies employing formaldehyde stabilization of the collagen molecule through the introduction of methylene cross bridges between the α chains (Veis and Drake, 1963) suggested a method whereby different collagen molecules might be separated (Nold *et al.*, 1970). In the present study formaldehyde stabilization of native collagen in dilute solutions has been successfully used to separate two different collagen molecules from lathyritic chick xiphoid cartilage. The results provide direct support for the suggestion (Miller and Matukas, 1969) that cartilage collagen contains two distinct molecular species.

Materials and Methods

White Leghorn chickens, 1 week of age, were placed on a diet of corn mash containing 0.1% β-aminopropionitrile fumarate to render them lathyritic. At 2.5 weeks of age the animals were killed, and the xiphoid cartilages were dissected free of surrounding tissues and diced. The tissue was extracted overnight in the cold in 0.4 ionic strength phosphate buffer, pH 7.6, with gentle shaking. The extract was then filtered through cheesecloth and clarified by centrifugation. The collagen was precipitated by adding NaCl to a final concentration of 20% and letting the solution stand overnight. The precipitate was collected by centrifugation, redissolved in phosphate buffer, and clarified by centrifugation. The second precipitation was done by adding cold 100% ethanol to a final concentration of 14% and letting the solution stand overnight. The precipitate was dissolved in phosphate buffer and precipitated a third time by dialysis against 0.01 M Na_2HPO_4 with two changes over 48 hr. After the last neutral precipitation, the collagen was solubilized in 0.1 M acetic acid and precipitated by adding NaCl to a final concentration of 7%. The precipitate was dissolved in neutral phosphate buffer and precipitated a final time by dialysis against 0.01 M Na_2HPO_4. The collagen was then solubilized in and dialyzed against 0.1% acetic acid, lyophilized, and stored at $-40°$. Up to seven phosphate extractions were performed on each batch of tissues; each extract was purified separately in a similar manner. In a typical extraction the following yields of purified collagen were obtained from five sequential extractions of 19 g of cleaned wet xiphoid cartilages (80 animals): 44; 34; 30; 20; 18 mg.

Chromatography of the purified heat-denatured collagen was performed on 0.9 × 5 cm columns of carboxymethyl-(CM) cellulose in 0.06 M sodium acetate buffer, pH 4.86, with a linear gradient of NaCl from 0 to 0.10 M over a total volume of 200 ml (Piez *et al.*, 1963). By miniaturization of the column and use of a micro flow cell in the spectrophotometer, it has been possible to examine 1- to 2-mg quantities of purified collagen.

Polyacrylamide disc gel electrophoresis was done according to the method of Stark and Kühn (1968).

Artificial intramolecular cross-links were introduced into the collagen molecule by treating solutions of collagen at concentrations of 0.5 mg/ml with 3.7% formaldehyde at pH 3.6 according to the procedure described by Veis and Drake (1963). Cross-linked preparations were examined by ion-exchange chromatography on CM-cellulose as described above and by molecular sieve chromatography on a 2 × 110 cm column of Agarose (Piez, 1968).

Amino acid analyses were performed using the single-column method on an automatic instrument (Beckman 102C) modified for high-speed analyses (Miller and Piez, 1966). Samples were hydrolyzed in constant-boiling HCl at 108° for 24 hr in a tube sealed under nitrogen. Correction factors for the labile amino acids (serine, threonine, methonine, and tyrosine) and for the incomplete release of valine were applied as previously determined (Piez *et al.*, 1960).

Carbohydrates were analyzed on a Perkin-Elmer Model 900 gas–liquid chromatograph equipped with dual flame ionization detectors and columns of 3% OV-1 and 3% OV-17 coated on 60–80 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). Samples were heated for 16 hr at 65° in 5.0 M methanolic HCl and then were per(trimethylsilyl)ated with Tri-Sil (Pierce Chemical Co., Rockford, Ill.) and injected at 120°; the temperature was programmed for a raise of 5° per min to 265° (Clamp *et al.*, 1967). They were also assayed for carbohydrate by the anthrone method (Dische, 1962).

Electron Microscopy of SLS.[1] Native collagen from chick skin and the third extract of xiphoid cartilage were dissolved in 0.05 M acetic acid, pH 3.5, and dialyzed against 0.4% salt-free ATP in the cold at pH 2.5 for 18 hr. The resultant cloudy suspension of segment long spacings (SLS) was applied to collodion–carbon film and stained with uranyl acetate, pH 3.8. These preparations were examined in an RCA EMU 3G electron microscope.

Results

Carboxymethylcellulose Chromatography of Denatured Collagen. The heterogeneity of cartilage collagen is apparent in CM-cellulose chromatograms of the denatured collagens obtained from the serial neutral phosphate extracts. That fraction obtained from the first phosphate extract demonstrates significant heterogeneity in the α1 region (Figure 1A). The α2 chain appears homogeneous. Because of the lathyritic state of the animals very little β_{11} or β_{12} is present. Although inhomogeneity of the α1 chains can occur for reasons other than intrinsic heterogeneity of the collagen molecules (Piez *et al.*, 1966; Kang *et al.*, 1969), the chromatogram from the first extract indicates an unusual distribution of chains in that there is 4 times as much α1 as α2. If cartilage collagen were comprised mainly of $(\alpha 1)_2\alpha 2$ molecules as in skin and bone, only twice as much α1 as α2 would be expected.

The chromatogram of purified collagen from the third phosphate extract (Figure 1B) provides even more convincing evidence of an unusual situation in cartilage and suggests a composition predominantly of α1 chains in that the α1

[1] Abbreviation used is: SLS, segment long spacings.

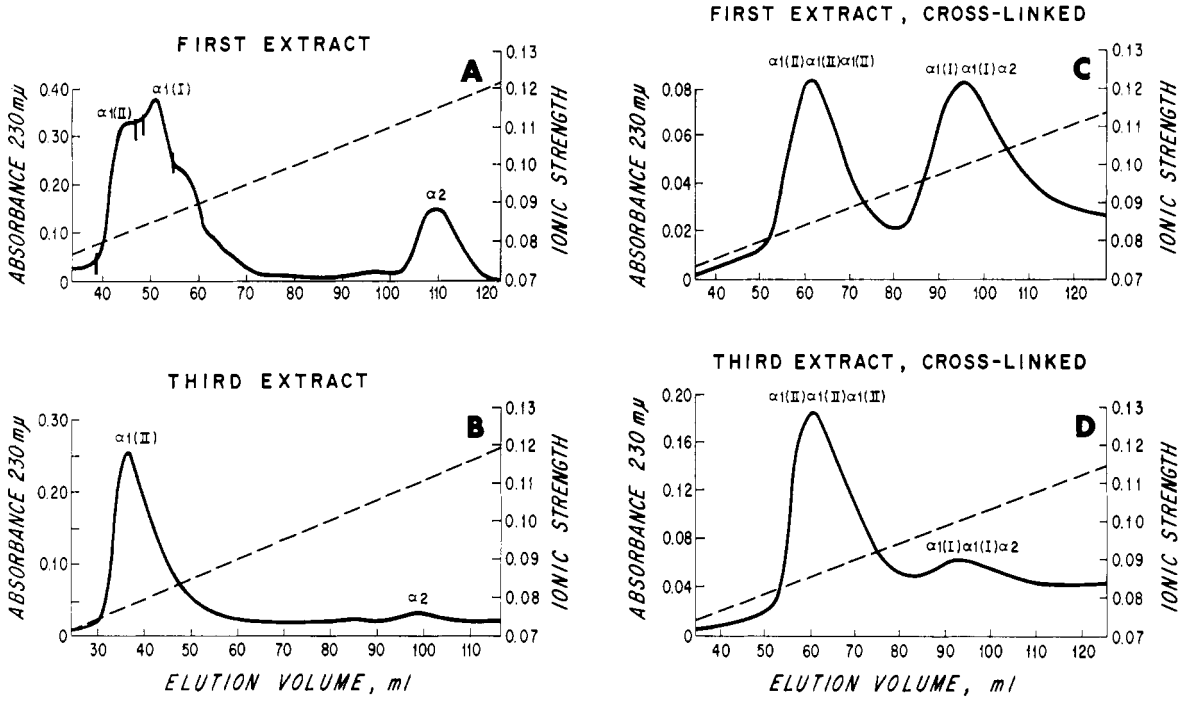

FIGURE 1: Chromatograms from carboxymethyl (CM) cellulose of denatured cartilage collagen and formaldehyde cross-linked cartilage collagen. The ionic strength of the salt gradient is indicated by the dotted line. The chromatogram of the first extract (A) shows heterogeneity of $\alpha 1$ chains and a homogeneous $\alpha 2$. Fractions for amino acid analysis were taken from peaks 1 and 2 of the $\alpha 1$ region as indicated by the vertical lines. The identification of the two different $\alpha 1$ chains, $\alpha 1$ type I and $\alpha 1$ type II, is based on amino acid analyses and the terminology is that introduced by Miller and Matukis (1969) (see text). The chromatogram of the third extract (B) shows a homogeneous $\alpha 1$ (II) chain and very little $\alpha 2$. When the collagen in these two extracts is stabilized by intramolecular formaldehyde cross-linkages and then chromatographed on CM-cellulose the chromatograms illustrated in C and D are obtained. In the first extract (C) two different molecules are present in nearly equal amounts. In the third extract (D) predominantly one molecule is obtained. The probable chain compositions of the two molecules judged by amino acid analysis and chromatographic behavior are indicated.

peak is homogeneous and the ratio of $\alpha 1$ to $\alpha 2$ is 30. This large excess of $\alpha 1$ chains suggests that the predominant collagen in the third extract has a chain composition of $(\alpha 1)_3$.

The chromatographic pattern of the purified collagen from the second phosphate extract is intermediate in appearance between the first and third extracts, and the ratio of $\alpha 1$ to $\alpha 2$ is 16.2. The chromatograms of the purified collagens from the fourth through seventh extracts were similar to the third.

Chromatography of purified neutral phosphate extracts of xiphoid cartilage from nonlathyritic chicks revealed a pattern similar to that seen in the later phosphate extracts of cartilage from lathyritic animals (unpublished data).

Polyacrylamide disc gel electrophoresis of the peptide material represented by all major peaks from the chromatograms indicated that they consisted of pure α chains.

Chromatography of Formaldehyde Cross-linked Collagen. The chromatograms of CM-cellulose of cartilage collagen which has been stabilized by intramolecular formaldehyde cross-linkages clearly demonstrate the presence of two different molecular species (Figure 1C,D). In addition the chromatograms show that the relative proportion of the two collagen molecules present in the first extract (Figure 1C) is significantly different from that present in the third (Figure 1D).

The area ratio of the two peaks of the first phosphate extract is 0.9:1.0 (Figure 1C) and the elution position of the first molecule is similar to that for an isolated $\alpha 1$ chain. The elution position of the second peak slightly precedes that of a β_{12} molecule and is essentially identical with that found for the formaldehyde cross-linked $(\alpha 1)_2\alpha 2$ molecule in guinea pig skin (Nold *et al.*, 1970). The chromatograms of the cross-linked collagens from the third phosphate extract also reveal two molecules, but in this case they are present in a ratio of 7.7:1.0 (Figure 1D). The elution positions of the two peaks in the third extract are identical with those found in the first extract.

Chromatography on an Agarose column, which had been calibrated with the cyanogen bromide peptides of rat skin collagen, α chains, β components, and formaldehyde cross-linked guinea pig skin collagen (Nold *et al.*, 1970), all of whose molecular weights had been determined by the sedimentation equilibrium method (Yphantis, 1964), indicated that the molecular weight of peak 1 from the cross-linked cartilage collagen was 290,000.

Amino Acid Analyses. The amino acid compositions of the formaldehyde cross-linked cartilage collagens and native uncross-linked cartilage and skin collagens are reported in Table I. The amino acid analysis of purified native cartilage collagen from the later extracts, which has a chain composition of $[\alpha 1(II)]_3$, is clearly different from that of the native collagen from skin which has a chain composition of $[\alpha 1(I)]_2\alpha 2$ (Kang *et al.*, 1969). The amino acid analyses of the two cross-linked cartilage collagens indicated that the

TABLE I: Amino Acid Composition of Native and Formaldehyde Cross-linked Cartilage Collagens and Native Skin Collagen.[a]

	Native Cartilage[b]	Cross-linked Cartilage[c]		Native Skin[d]
	$[\alpha1(II)]_3$	$[\alpha1(II)]_3$	$[\alpha1(I)]_2\alpha2$	$[\alpha1(I)]_2\alpha2$
4-Hydroxyproline	99	88	88	106
Aspartic acid	43	45	48	45
Threonine	24	28	24	19
Serine	24	41	43	29
Glutamic acid	87	94	85	73
Proline	117	105	119	118
Glycine	331	327	315	332
Alanine	102	103	110	116
Valine	21	21	23	18
Methionine	9.8	11	6.0	7.4
Isoleucine	9.1	9.2	12	10
Leucine	27	26	26	24
Tyrosine	1.5	2.7	3.4	1.6
Phenylalanine	14	15	15	12
Hydroxylysine	22	19	12	6.9
Lysine	17	16	23	27
Histidine	3.2	5.4	7.2	4.2
Arginine	52	46	46	51
Proline % hydroxylated	45.8	45.6	42.5	47.3
Lysine % hydroxylated	56.4	54.3	34.3	20.4

[a] Residues per 1000 total residues. The values represent the averages of at least two analyses. [b] Extracts 3 and 4. [c] Peaks 1 and 2, respectively, from cross-linked extracts 1 and 3. [d] Kang *et al.* (1969).

TABLE II: Amino Acid Composition of the α Chains Derived from Denatured Cartilage Collagens.[a]

	$\alpha1$[b]			
	Peak 1	Peak 2	$\alpha1(II)$[c]	$\alpha2$[d]
4-Hydroxyproline	99	95	100	90
Aspartic acid	44	45	44	50
Threonine	25	22	25	21
Serine	24	25	26	32
Glutamic acid	83	82	85	67
Proline	113	117	113	115
Glycine	338	340	336	339
Alanine	107	117	105	102
Valine	19	17	19	32
Methionine	11	9.3	11	2.6
Isoleucine	7.8	7.3	7.6	19
Leucine	25	22	25	31
Tyrosine	2.3	2.7	1.5	3.2
Phenylalanine	14	14	14	14
Hydroxylysine	20	15	22	13
Lysine	19	22	16	18
Histidine	2.1	2.5	2.3	7.8
Arginine	49	50	49	49
Proline % hydroxylated	46.6	44.8	46.9	43.9
Lysine % hydroxylated	51.3	40.5	57.9	41.9

[a] Residues per 1000 residues. The values represent the averages of at least two analyses. [b] Extract 1 in which heterogeneity of $\alpha1$ was found on CM-cellulose chromatography. Peak 1 represents material obtained from between the first two vertical lines in Figure 1A; peak 2, from between the last two vertical lines. [c] Extracts 2 through 5. [d] Extract 1.

first peak of material to elute from the CM-cellulose was almost identical with the native $[\alpha1(II)]_3$ of cartilage. The second peak of cross-linked material to elute has a composition which more closely resembles the native collagen from skin and its chain composition is assigned on this basis and on its elution position. The increase in the amino acids threonine, serine, glutamic acid, and histidine and the decrease in proline, hydroxyproline, and glycine in the cross-linked preparations are unexplained.

The composition of the isolated α chains, reported in Table II, indicates that at least three different α chains are present in the cartilage collagens. The $\alpha2$ chain, which was present in significant amounts only in the first extract, is clearly different from the $\alpha1$ chains and the composition of the $\alpha2$ chain from cartilage is similar to the $\alpha2$ obtained from skin (Kang *et al.*, 1969). Analysis of the $\alpha1$ chains from the first extract, in which a heterogeneous population was found on chromatography, indicated that two different forms of $\alpha1$ were present. The two $\alpha1$ chains in the first extract have not been isolated in pure form and the identification of $\alpha1(I)$ and $\alpha1(II)$ indicated in Figure 1A is tentative. It is clear, however, that the first of the two $\alpha1$ chains to elute from the CM-cellulose, peak 1, had a composition different from that of peak 2. Moreover the composition of peak 1 is nearly identical with that obtained for the $\alpha1$ chains from the second through fifth extracts in which a large excess of homogeneous $\alpha1$ chains was found on chromatography. The $\alpha1$ chain from these later extracts is the $\alpha1(II)$ and is clearly different from the $\alpha1(I)$ of chick skin (Kang *et al.*, 1969).

Carbohydrate Composition. Analysis of the carbohydrate content of the purified $\alpha1$ from the third and fifth extracts indicated 5.5% carbohydrate by weight. Galactose and glucose were the only hexoses present and these were found in a molar ratio of 2:1. No pentoses or hexosamines were detected.

Band Pattern of Segment Long Spacing. Electron microscopic examination of SLS of collagen from the third cartilage extract as compared with that from chick skin revealed significant differences. As shown in Figure 2 there are definite and reproducible differences in the $\alpha1$-CB-8 region of the cartilage collagen molecule as compared with the same region of chick skin collagen molecules. The overall pattern of intensity and sharpness in this region of cartilage collagen SLS is quite characteristic. Perhaps the clearest difference is noted in the location emphasized by drawn-in lines in Figure 2. All the SLS in every field of cartilage collagen revealed this charac-

teristic picture. There appear to be less well-defined differences in band density and width in other regions; however, at this time these are not quite as well defined. The length of the segments appears to be identical for the two tissue collagens.

Histological Examination of Xiphoid Cartilage. Cross sections through the long axis of the well-cleaned xiphoid cartilage examined in the light microscope revealed two distinct regions: the metachromatic matrix and the non-metachromatic perichondrium. The perichondrium is about 10–15 μ thick and is present around the entire periphery of the tissue. The collagen in the perichondrium was distinctly birefringent when viewed through crossed polarizers; the collagen fibers within the metachromatic matrix were only weakly birefringent.

Discussion

These studies indicating that lathyritic cartilage contains two distinct collagen molecules between which three different α chains are distributed are fully in accord with the conclusions of Miller and Matukas (1969).

In order to conform to the terminology introduced by Miller and Matukas the three chains described in the present study have been labeled α1 type I, α1 type II, and α2. Identification of the α2 chain by CM-cellulose chromatography was unambiguous and presumably identical with that found by Miller and Matukas (1969). The two types of α1 chain were characterized and distinguished from each other by Miller and Matukas (1969) on the basis of their cyanogen bromide derived peptides and differences in amino acid composition of one of them, CB-2. The α1 type I seems very similar to the α1 of skin (Kang *et al.*, 1969) and bone (Miller *et al.*, 1967) and the several small differences found in our study are probably related to the incomplete separation from α1 type II during chromatography. The α1 type II is easily detectable in the first extract and is present in large excess in the later extracts. In the first extract, where the two α1 chains are present in nearly equal amounts, the first α1 to elute from the CM-cellulose is α1 type II and the second type, α1 type I.

The chain composition of the two molecules as determined here is $[\alpha1(II)]_3$ and $[\alpha1(I)]_2\alpha2$. The predominant molecule in the later extracts is clearly $[\alpha1(II)]_3$ and accounts for more than 90% of the extracted fractions. The more typical molecule, $[\alpha1(I)]_2\alpha2$, is present in significant quantities only in the first extract. Its chain composition is best indicated by its similarity to the $(\alpha1)_2(\alpha2)$ of guinea pig skin collagen under conditions of formaldehyde cross-linkage and CM-cellulose chromatography (Nold *et al.*, 1970). The amino acid analyses are also consistent with this chain composition.

The relatively high amount of carbohydrate, 5.5% by weight, approximately 30 residues per α chain, which is covalently bound to the α1 type II illustrates another difference between this molecule and the characteristic α1 chain of skin where there is 0.5% by weight of carbohydrate (A. H. Kang, unpublished data). From the work of Butler and Cunningham (1966) on rat skin collagen, it would seem likely that this carbohydrate is linked to the α chain at a hydroxylysyl residue. The high percentage of hydroxylated lysyl residues in cartilage collagen is consistent with the increased sugar content.

It is of interest that up until now SLS patterns of a wide range of collagens from both vertebrate and invertebrate tissues were thought to be very similar even though there are significant and numerous differences in amino acid composition (Gross, 1963; Olsen, 1967; Nordwig and Hayduk, 1969). In addition, the SLS formed from the reconstituted pure α1 and α2 chains have been called identical (Tkocz and Kühn, 1969). Although there are significant differences in amino acid composition between chick cartilage collagen $[\alpha1(II)]_3$ and skin collagen $[\alpha1(I)]_2\alpha2$, these are relatively small, yet the SLS band patterns are clearly different. Since uranyl acetate at acid pH presumably stains only acidic residues the unusual band pattern should assist us in understanding the molecular structure. Detailed analyses of the SLS structure of cartilage collagen as well as an examination of physical chemical properties will be reported in greater detail at another time.

The ratios of the three α chains of cartilage collagen $\alpha1(I):\alpha1(II):\alpha2$, observed by us in the first and third extracts, were 2:2:1 and 2:28:1, respectively, as compared with 2:1:1 observed by Miller and Matukas (1969) who used a different extraction procedure. The slightly alkaline phosphate buffer (pH 7.6, ionic strength 0.4) used by us solubilizes both the lathyritic collagen and the acid mucopolysaccharides in the cartilage matrix. During subsequent purification of the collagen by differential precipitation at neutrality, the acid

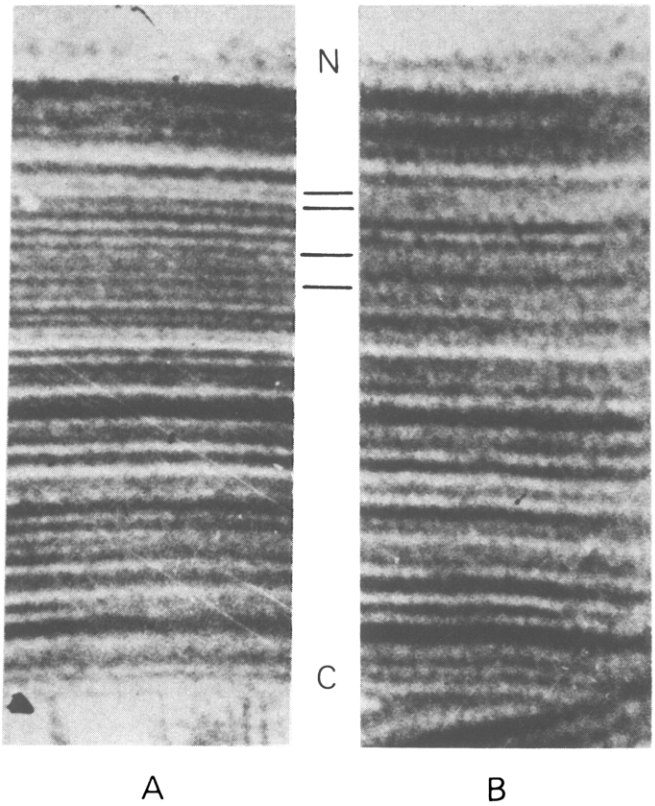

FIGURE 2: Electron micrographs of segment long spacings (SLS) of: A, chick xiphoid cartilage collagen, and, B, chick skin collagen. Stained with 12 mM uranyl acetate, pH 3.8. Lines drawn between segments indicate the loci at which clear and reproducible band differences appear. Other differences in staining density elsewhere in the pattern may also be discerned but are not as sharply defined. N, NH_2 terminus of molecule, and C, COOH terminus. Magnification 316,666×.

mucopolysaccharides remain in solution and the problems of coprecipitation encountered by Miller and Matukas are avoided. In addition considerable enrichment of the $[\alpha 1(II)]_3$ fraction was obtained by repetitive extractions which greatly improved the yield over that reported by Miller and Matukas (1969).

The change in proportion of the two cartilage collagens which are solubilized by the successive phosphate extractions may simply indicate that the $[\alpha 1(I)]_2\alpha 2$ molecule is more soluble than $[\alpha 1(II)]_3$ and that nearly all of the extractable $[\alpha 1(I)]_2\alpha 2$ is removed in the first extraction. An alternative explanation is that the two molecules are not randomly distributed throughout the cartilage matrix, but instead the $[\alpha 1(I)]_2\alpha 2$ is predominantly located in the perichondrium and the $[\alpha 1(II)]_3$ is the major component of the matrix proper. From the histological observations in the present study it is clear that the perichondrium is not removed from well-cleaned xiphoid cartilage. It is quite conceivable that the superficial position of the perichondrium would render its soluble collagen more readily extractable whereas the collagen lying with the cartilaginous matrix would gradually be solubilized as the matrix was progressively dissolved. The implication of this latter explanation is that the collagen in the cartilaginous matrix is not heterogeneous but, in fact, consists of only one kind of collagen, the $[\alpha 1(II)]_3$.

The fact that the cartilage collagen molecule is made up of three α chains from a distinctly different genetic locus (Miller and Matukas, 1969) from the α chains present in bone has obviously important implications for osteogenesis. In the embryo the distribution and shape of many bones are initially established by a cartilaginous anlage. With ossification the cartilage matrix is removed and replaced with a different form of collagen, $[\alpha 1(I)]_2\alpha 2$, characteristic of mature bone, tendon, and skin. Because of the host of developmental defects which affect skeletal growth and development as well as pathological conditions involving skeletal tissues in the adult, future investigations of cartilage and other soft tissue collagens will be of interest.

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