Isolation and Characterization of a Collagen from Chick Cartilage Containing Three Identical α Chains*

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ABSTRACT: Collagen extracted with 1.0 M NaCl at neutral pH from the sternal cartilages of lathyritic chicks has been examined in regard to chain composition, amino acid composition, carbohydrate content, and thermal stability. Approximately 30% of the total tissue collagen could be brought into solution by employing four successive, 3-day extraction periods. Partial purification of the extracted collagen was achieved by passing the extracts at reduced ionic strength over a column of diethylaminoethylcellulose in order to remove proteoglycans. Carboxymethylcellulose chromatography of the collagen obtained in the initial extraction period revealed an $\alpha 1$ to $\alpha 2$ ratio of approximately 10:1, a ratio far in excess of that required for molecules of the chain composition $\alpha 1_2\alpha 2$. These results are in accordance with previous findings which indicate that the excess $\alpha 1$ chain can be explained by the presence of an additional $\alpha 1$ chain, $\alpha 1$(II), presumably derived from molecules of the chain composition $[\alpha 1(II)]_3$. In support of this concept, continued extraction of cartilage preparations solubilizes collagen with an increasing $\alpha 1$ to $\alpha 2$ chain ratio and collagen extracted during third and fourth extraction periods contains no detectable $\alpha 2$ chain. Examination of the cyanogen bromide peptides of the $\alpha 1$ fraction of third and fourth extracts indicates that $\alpha 1$(II) is the only chain present in these extracts and therefore must be derived from molecules of the chain composition $[\alpha 1(II)]_3$. Calculations based on the $\alpha 1$ to $\alpha 2$ ratios and the amount of collagen obtained in each extract indicate that approximately 90% of the extracted collagen can be accounted for as $\alpha 1$(II). Amino acid analyses of the $\alpha 1$(II) chain obtained in third and fourth extracts and comparison to the composition of $\alpha 1$(I) confirm the previous suggestion that synthesis of $\alpha 1$(II) is directed by a different structural gene from that responsible for $\alpha 1$(I) synthesis. The two types of $\alpha 1$ chain exhibit large differences in the content of glutamic acid, alanine, and leucine. In addition, $\alpha 1$(II) could be readily differentiated from $\alpha 1$(I) on the basis of a fivefold increase in the extent of hydroxylation of lysyl residues and a ninefold increase in the content of hydroxylysine-linked carbohydrate. Thermal denaturation studies in dilute acid solution indicate that molecules of the chain composition $[\alpha 1(II)]_3$ are somewhat less stable than molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ under the conditions of the study.

These results provide yet another means of differentiating molecules comprised of the $\alpha 1$(II) chain from those containing $\alpha 1$(I).

It is generally agreed that the native collagen molecule has a coiled-coil, triple-chain structure. Biochemical studies on soluble collagens from various species further indicate that different organisms have met the challenges of selection and adaptation not only through modifications in the primary structure of individual α chains within the collagen molecule, but through the elaboration of multiple genes for collagen synthesis as well. For example, in most of the vertebrate collagens thus far examined, the amino acid composition (and therefore the primary structure) of one of the three chains (the $\alpha 2$ chain) differs significantly from that of the other two (the $\alpha 1$ chains) and the two types of α chains are readily separable by chromatography on CM-cellulose columns. Collagens of the chain composition ($\alpha 1_2\alpha 2$) include rat skin (Piez *et al.*, 1963), dogfish skin (Lewis and Piez, 1964), human skin (Bornstein and Piez, 1964), chick bone (Miller *et al.*, 1967), and chick skin (Kang *et al.*, 1969c). It may thus be concluded that at least two structural genes code for collagen synthesis in these tissues from these species. On the other hand, there is one example of a collagen which may be derived from the operation of three structural genes in that all three α chains of codfish skin collagen differ significantly in amino acid composition and are readily separated by CM-cellulose chromatography (Piez, 1965). Furthermore, there is evidence suggesting that all three α chains in the collagens of some of the lower vertebrates and invertebrates are identical (Pikkarainen, 1968; Pikkarainen *et al.*, 1968; Nordwig and Hayduk, 1969), and that more primitive organisms may utilize only one structural gene for collagen synthesis.

This survey of collagens from organisms in various plyla clearly indicates that the genetic information for collagen synthesis may be expanded as one moves higher on the evolutionary scale. With regard to collagen synthesis in different tissues of a given organism, comparison of the CNBr peptides derived from the $\alpha 1$ and $\alpha 2$ chains of rat skin and tendon collagens (Bornstein, 1969) and from the $\alpha 1$ and $\alpha 2$ chains of chick bone and skin collagens (Miller *et al.*, 1969; Lane and Miller, 1969; Kang *et al.*, 1969a,b) indicated that, for a given species, collagen synthesis was directed by the same set of genes in the tissues examined. However, recent work in our laboratory (Miller and Matukas, 1969, 1970) has demonstrated a tissue-specific synthesis of collagen in chick cartilages, which was manifest by the presence of a large excess of $\alpha 1$ chains (over that required for a chain composition of ($\alpha 1_2\alpha 2$)) when collagen extracted from sternal cartilage was chromatographed on CM-cellulose. Cleavage of the $\alpha 1$ fraction with CNBr and isolation of the resulting peptides revealed that the lack of stoichiometry was due to the presence of an additional type of $\alpha 1$ chain which is not found in collagens extracted from chick bone or skin. The $\alpha 1$ chain which is apparently restricted to

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cartilage collagen has been termed $\alpha 1$(II) to distinguish it from the $\alpha 1$(I) chains common to bone, skin, and cartilage collagens. It was further proposed (Miller and Matukas, 1970), based on the relative proportions of $\alpha 1$(I), $\alpha 1$(II), and $\alpha 2$ chains in total cartilage collagen, that a sizeable proportion of the $\alpha 1$(II) chains were assembled into molecules having the chain composition, $[\alpha 1(II)]_3$.

The present paper describes the extraction and purification of cartilage collagens and the isolation of molecules of the composition, $[\alpha 1(II)]_3$, and presents some of the physical and chemical properties of the $\alpha 1$(II) chain and the molecules comprised of the $\alpha 1$(II) chain.

Materials and Methods

Collagen Extraction and Purification. Collagen was extracted from the sternal cartilages of 3-week-old chicks which had been raised from the time of hatching on a commercial ration containing 0.1% β-aminopropionitrile fumarate (Miller *et al.*, 1967). The cartilages were prepared for extraction as previously described (Miller and Matukas, 1969), and in the present study the cartilage slices were extracted with 1.0 M NaCl (0.05 M Tris, pH 7.5) at 4° for four successive intervals of 3 days each. In general, preparations of cartilage collagen involved extraction from the sternal cartilages of 300 chicks and the amount of solvent utilized in the first 3-day extraction period was 250 ml. The amount of solvent employed for the second, third, and fourth extracts was reduced to 100 ml. All extracts were clarified by centrifugation at 50,000*g* at 4°.

In a previous study (Miller and Matukas, 1969) the desirability of removing proteoglycans from the cartilage extracts was pointed out and selective precipitation of the proteoglycan molecules complexed with cetylpyridinium chloride was employed. In the present study, proteoglycans were more conveniently separated from the soluble cartilage collagen by passing the extracts over DEAE-cellulose. For this purpose, each extract was dialyzed overnight at 4° against 100 volumes of 0.2 M NaCl (0.05 M Tris, pH 7.5). During dialysis, the solution within the dialysis tubing remained clear. Following dialysis, 50-ml aliquots of each extract were applied to a 2.5 × 15 cm column of DEAE-cellulose (Whatman, microgranular DE-32, capacity, 1.0 mequiv/g) equilibrated with 0.2 M NaCl (0.05 M Tris, pH 7.5). The column temperature was maintained at 5° in order to avoid denaturation of the extracted collagen and the column was eluted at a flow rate of 100 ml/hr. The column effluent was monitored and recorded as described previously (Miller *et al.*, 1967, 1969) and the effluent was collected in flasks placed in an ice bath. Following application of the sample to the column, elution with 0.2 M NaCl (0.05 M Tris, pH 7.5) was continued until no further ultraviolet-absorbing material was detected in the effluent. At this time, the eluting solvent was changed to 1.0 M NaCl (0.05 M Tris, pH 7.5) and elution with the latter buffer was continued until an additional peak was eluted from the column. The column was reequilibrated with the starting buffer and was ready for reuse.

As shown in Figure 1, DEAE-cellulose chromatography of neutral-salt extracts of cartilage resulted in the fractionation of the total extract into two major components. Aliquots of the effluent from both peaks were dialyzed against water and lyophilized, and amino acid analyses (see below) were performed on the dried material after hydrolysis in 8 M HCl under nitrogen at 95° for 3 hr to minimize hydrolytic destruction of hexosamines (Tsiganos and Muir, 1969). The results

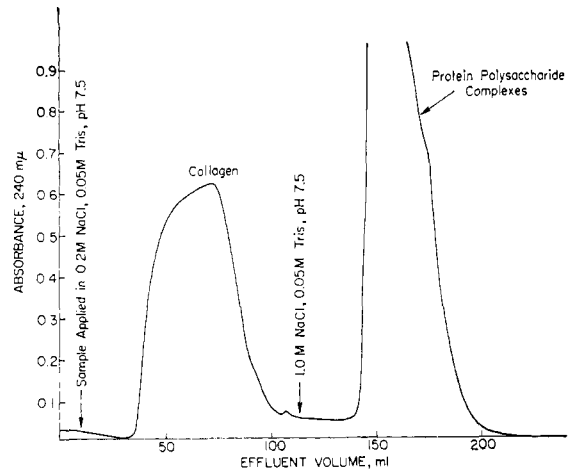

FIGURE 1: DEAE-cellulose chromatography of a 50-ml aliquot of a first extract of sternal cartilages. The sample is applied to the column after dialysis against buffered 0.2 M NaCl and elution with the same buffer is continued until the unretained collagen-containing fraction is eluted. At the designated time, elution with buffered 1.0 M NaCl is begun in order to remove acidic protein polysaccharide complexes from the column.

of the analyses showed that the collagen in the extracts had been eluted in the first unretained fraction from the DEAE column. Moreover, this fraction was entirely free of proteoglycans as judged by the absence of glucosamine and galactosamine. On the other hand, the fraction eluted by 1.0 M NaCl contained neither hydroxyproline nor hydroxylysine, but contained trace amounts of glucosamine and large amounts of galactosamine. These results indicated that DEAE-cellulose chromatography under the conditions outlined above provided a rapid and efficient method for separating collagen from proteoglycans in cartilage extracts.

Additional purification of the cartilage collagen was achieved by precipitation of the collagen contained in the unretained DEAE-cellulose fraction during dialysis against 0.02 M Na_2HPO_4. The precipitate was collected by centrifugation, redissolved in 0.5 M acetic acid, dialyzed against a large volume of the same solvent, and lyophilized. Amino acid analyses of the lyophilized material indicated that it was essentially pure collagen and no further purification steps were employed.

CM-cellulose Chromatography. Fractionation of the cartilage collagen on CM-cellulose was achieved essentially as described previously (Piez *et al.*, 1963; Miller *et al.*, 1967). In the present study, however, it was noted that the lyophilized cartilage collagen was more readily dissolved in the starting buffer, the solutions appeared to be more stable, and better recovery of the chromatographed collagen was achieved if the starting buffer contained urea. Therefore, the conditions for CM-cellulose chromatography were modified by reducing the ionic strength of, and adding urea to, the eluting buffers. Weighed samples of cartilage collagen (150–200 mg from first extracts, and 45–55 mg from subsequent extracts) were dissolved at 5° at a concentration of 2 mg/ml in 0.02 M (Na^+) sodium acetate (pH 4.8) containing 1.0 M urea (the starting buffer). If the sample contained more than 100 mg of collagen the column dimensions were 2.5 × 10 cm whereas for smaller samples containing up to 60 mg of collagen, the column dimensions were 1.8 × 10 cm. The larger columns were operated at a flow rate of 200 ml/hr and the flow rate in the smaller columns

was 100 ml/hr. For the larger columns, elution was carried out by means of a linear gradient established by means of a two-chamber constant-level device containing 500 ml of starting buffer in the first chamber and 500 ml of limit buffer (0.02 M (Na^+) sodium acetate containing 0.1 M NaCl and 1.0 M urea, pH 4.8) in the second chamber. For the smaller columns, the quantities of buffer used in each chamber were halved. Recovery of collagen chromatographed on the columns was estimated after planimetry of the various peaks (Miller *et al.*, 1967).

CNBr Cleavage and Chromatography of CNBr Peptides. In the present study, cleavage at the methionyl residues of the α chains of cartilage collagen and chromatography of the resulting peptides were employed to determine the relative amounts of α1(I) and α1(II) which appeared in CM-cellulose chromatograms. CNBr cleavage of the α chains was performed under conditions identical with those previously utilized for the collagen extracted from chick bone (Miller *et al.*, 1969; Lane and Miller, 1969). Following the 4-hr reaction period, the total CNBr digest was lyophilized and redissolved in a minimal amount of 0.1 N acetic acid, and 1-ml aliquots of the redissolved peptides were chromatographed on a 1.7 × 80 cm column of Bio-Gel P-4 as described previously (Lane and Miller, 1969). Subsequently, phosphocellulose chromatography (Miller and Matukas, 1969) was performed on those peptides which had been eluted in the exclusion volume of the P-4 column. This procedure eliminated peptides of mol wt 2000 and lower from the mixture applied to the phosphocellulose column and considerably simplified the phosphocellulose elution patterns. The proportions of α1(I) and α1(II) could then be estimated directly from the phosphocellulose pattern by determination of the amounts of α1(I)-CB2 and α1(II)-CB2 (Miller and Matukas, 1969).

Molecular Sieve Chromatography. The α chains and CNBr peptides eluted from ion-exchange columns were desalted on Bio-Gel P-2 columns as previously described (Miller *et al.*, 1969; Lane and Miller, 1969). In addition aliquots of the components eluted from CM-cellulose columns were chromatographed on a calibrated 590-ml column of agarose beads (Bio-Gel A-1.5) in order to determine the molecular weight distribution of the material in each peak (Piez, 1968; Miller *et al.*, 1969).

Carbohydrate Determinations. Total protein-bound hexose of the purified α1(II) chain (5-mg samples) from cartilage collagen was determined by the orcinol–sulfuric acid method (Winzler, 1955). For these analyses, a standard curve utilizing varying dilutions of a solution containing galactose and glucose in a 2:1 molar ratio was employed. Qualitative identification of the hexoses was achieved by chromatography on Whatman No. 1 paper in 1-butanol–pyridine–water (6:4:3, v/v) after hydrolysis of 5-mg aliquots of α1(II) in 3 ml of 2 N HCl under N_2 for 2 hr at 110° (Butler and Cunningham, 1966). Hydrolysates representing 5 mg of collagen were applied at room temperature to a 1 × 10 cm column of Dowex 50 (H^+ form) and two column volumes of water were used for elution. The eluent was lyophilized and redissolved in 1 ml of water, and approximately 20 μl of the sample was applied along with appropriate standards in a similar volume to the paper. After development the chromatograms were sprayed with 3% *p*-anisidine hydrochloride in 1-butanol (Mann Research Laboratories, Inc.) for detection of hexoses.

Further aliquots of α1(II) (5 mg) were treated at pH 3.8 with 0.025 M periodic acid at room temperature for 4, 8, and 12 hr in the dark (Butler and Cunningham, 1966). After removal of excess periodate by gel filtration on a 1 × 20 cm column of Bio-Gel P-2, the samples were hydrolyzed under N_2 in 3 ml of 6 N HCl at 108° for 24 hr and 1-mg aliquots were utilized to determine by amino acid analysis the amount of hydroxylysine remaining after periodate treatment. In addition, 5-mg samples of α1(II) were hydrolyzed under N_2 in alkali-resistant tubes (Corning 7280) in 1 ml of 2 N NaOH at 108° for 24 hr (Spiro, 1969) to hydrolyze peptide bonds but preserve *O*-glycosidic linkages. The samples were neutralized with 0.5 M hydrochloric acid and 1-mg aliquots were submitted to amino acid analyses for the determination of glucosylgalactosylhydroxylysine[1] (Glu-Gal-Hylys), galactosylhydroxylysine[1] (Gal-Hylys), and hydroxylysine.

Amino Acid Analyses. With the exception of the special hydrolysis conditions outlined above, all protein and peptide samples were hydrolyzed under N_2 in 6 N HCl at 108° for 24 hr and prepared for analysis as previously described (Miller *et al.*, 1969). In all cases approximately 1 mg of sample was used for analysis on a single-column automatic amino acid analyzer equipped for high speed analyses (Miller and Piez, 1966). In the final calculations, losses of threonine, serine, and tyrosine, and incomplete release of valine were corrected for by the use of factors previously determined for collagen chains (Piez *et al.*, 1966).

Polarimetry. Collagen obtained in the third 1.0 M NaCl extract from sternal cartilage was dissolved at a concentration of 0.6 mg/ml in 0.5 M acetic acid. Optical rotation of the sample was measured at 313 mμ in a Cary Model 60 recording spectropolarimeter with a 1-cm jacketed cell with a mercury arc lamp as the light source. Thermal denaturation curves were obtained by a stepwise increase in temperature over the range 22–48°, with 30 min allowed for equilibration at each temperature. An identical study was also performed with acid-soluble collagen from chick bones. For the calculations of specific optical rotation, collagen concentrations in the solutions used for polarimetry were calculated by amino acid analyses.

Results

Solubility of Cartilage Collagen. Table I presents data with regard to the amount of purified collagen obtained during each of the four extraction periods from one representative preparation of sternal cartilages of lathyritic chicks. In this instance, cartilages had been prepared from 340 chicks sacrificed at 3 weeks of age. It is readily apparent that the yield of soluble collagen rapidly declines after the first extract and only four extraction periods were employed because of the minimal amounts of collagen obtained during the fourth extraction period. The combined extracts represent about 30% of the total tissue collagen. Collagen in cartilages from normal animals is completely insoluble in neutral salt solution (Miller and Matukas, 1969).

CM-cellulose Chromatography of Soluble Cartilage Collagen. Figure 2 depicts a CM-cellulose chromatogram of purified, heat-denatured sternal cartilage collagen obtained in the first neutral-salt extract. The major peaks were identified as α chains by molecular weight determinations on a calibrated agarose column. A small amount of what is presumed to be β_{12} can be observed preceding the α2 peak; however,

[1] Standards of glucosylgalactosylhydroxylysine and galactosylhydroxylysine were kindly provided by Dr. Sheldon R. Pinnell, Arthritis Unit, Massachusetts General Hospital.

TABLE I: Yield of Collagen from Lathyritic Chick Sternal Cartilages during Four Successive Extracts with Neutral-Salt Solutions (Cartilages from 340 Animals).

Extract	Collagen (mg)	% of Total Tissue Collagen
1	158	18
2	53	6
3	35	4
4	15	2

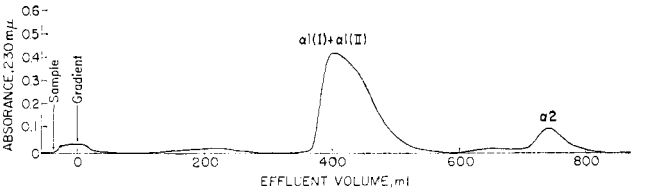

FIGURE 2: CM-cellulose elution pattern of 125 mg of denatured cartilage collagen obtained in a first extract. Chromatography is performed on a 2.5 × 10 cm column employing a linear gradient of ionic strength from 0.02 to 0.12 in 1.0 M urea at pH 4.8. (See text for details.)

the paucity of the material precluded further characterization. Planimetry of the curves revealed that approximately 95% of the collagen applied to the column had been recovered as the observed components. As previously shown for extracts of cartilage collagen (Miller and Matukas, 1969), the $\alpha 1:\alpha 2$ ratio in soluble cartilage collagen is greater than 2:1, due to the presence of the $\alpha 1$(II) chain presumably arising from molecules having the chain structure $[\alpha 1(II)]_3$. In this instance, Figure 2, the $\alpha 1:\alpha 2$ ratio is approximately 10:1 whereas our previous studies had indicated that this ratio reached 3:1 in similar extracts. It has subsequently been observed that the magnitude of the $\alpha 1:\alpha 2$ ratio in the first neutral-salt extract of cartilage collagen depends on the size of the cartilage slices used for extraction. If relatively large pieces of cartilage are extracted, the ratio of $\alpha 1:\alpha 2$ chains in the extracts falls in the range of 3–4:1. If, on the other hand, the sternal cartilages are sliced into very small pieces greatly increasing the ratio of newly exposed surface area to original surface area, the $\alpha 1:\alpha 2$ ratio in the collagen from the first neutral-salt extract approximates 10:1 as shown in Figure 2. These results suggest a nonrandom distribution of the collagen chains within the sternal cartilage and indicate that molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ lie near the periphery or perichondral surface of the tissue and that molecules of the chain composition $[\alpha 1(II)]_3$ are found in the deeper layers of the tissue.

It has also been noted that further extraction of the cartilage slices with neutral-salt solutions results in the solubilization of collagen containing even smaller proportions of the $\alpha 2$ chain. For instance, the $\alpha 1:\alpha 2$ ratio of the collagen obtained in the second extract is approximately 40:1. Finally, the collagen brought into solution in either the third or fourth extracts contains no detectable $\alpha 2$ chains as illustrated in Figure 3 for a third extract. Agarose molecular sieve chromatography showed that material in the peak depicted in Figure 3 was comprised entirely of α chains and planimetry of the curve indicated that all of the collagen applied to the column was recovered in the single peak.

These observations have been interpreted as indicating that early extracts yield a mixture of collagen molecules having the chain composition $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$ and that essentially all of the soluble $[\alpha 1(I)]_2\alpha 2$ molecules are brought into solution during the first and second extracts allowing one to extract uncontaminated $[\alpha 1(II)]_3$ molecules during subsequent extracts. This interpretation seems reasonable in light of the results presented above leading to the conclusion that molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ are located at the peripheral and more accessible regions of the tissue whereas molecules of the composition $[\alpha 1(II)]_3$ are localized in the deeper layers.

Although the $\alpha 1$ fraction of cartilage collagen obtained in third and fourth extracts routinely shows some chromatographic heterogeneity as illustrated in Figure 3, it has previously been shown that this type of heterogeneity may result from the presence of peptide-bound allysine on a certain proportion of the chains (Piez *et al.*, 1966) or from the loss of a small sequence of amino acids from the NH_2-terminal region of some of the chains (Kang *et al.*, 1969c). At present, insufficient data have been obtained to determine the cause of the chromatographic heterogeneity of the $\alpha 1$(II) chain in cartilage collagen. It may be related to one of the factors mentioned above or, alternatively, the chromatographic heterogeneity in this instance could arise from variability in location and nature of the relatively large amount of carbohydrate which is covalently bound to $\alpha 1$(II) (see below).

CNBr Cleavage and Chromatography of the CNBr Peptides of Cartilage Collagen α Chains. In order to provide further evidence for the conclusions with regard to the nature of the material chromatographing in the $\alpha 1$ region from various extracts, the $\alpha 1$ fractions from a first extract and a combined third and fourth extract were assayed as described above for the presence of CNBr peptides indicative of $\alpha 1$(I) and $\alpha 1$(II). The results for the first extract in which the $\alpha 1:\alpha 2$ ratio on CM-cellulose was 10:1 revealed that $\alpha 1$(II)-CB2 and $\alpha 1$(I)-CB2 were present in a ratio of approximately 3.8:1 indicating that the excess of $\alpha 1$ chains over that required for molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ could be accounted for by the presence of $\alpha 1$(II). In a similar study of the $\alpha 1$ fraction from third and fourth extracts in which no $\alpha 2$ chain was present in the CM-cellulose pattern, only $\alpha 1$(II)-CB2 could be detected in the phosphocellulose eluent indicating that the molecules brought into solution in later

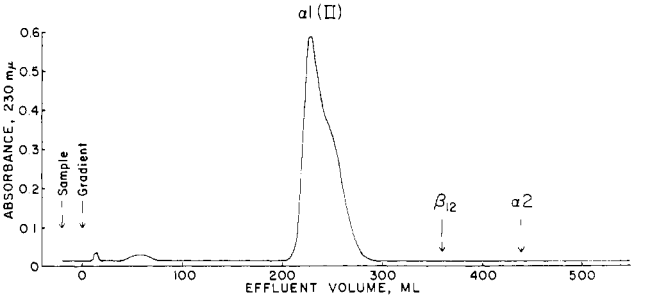

FIGURE 3: CM-cellulose elution pattern of 55 mg of denatured cartilage collagen obtained in a third extract. Chromatography is performed on a 1.8 × 10 cm column employing a linear gradient of ionic strength from 0.02 to 0.12 in 1.0 M urea at pH 4.8 as described in the text. Arrows designate the elution positions of β_{12} and $\alpha 2$ when samples of bone or skin collagen are chromatographed in the same system.

TABLE II: Amino Acid Composition of α1(I) and α1(II) in the Chick.

Amino Acid	Residues/1000 α1(I)[a]	α1(II)[b]
3-Hydroxyproline	1.0	2.2
4-Hydroxyproline	102	103
Aspartic acid	42	42
Threonine	19	26
Serine	29	26
Glutamic acid	78	87
Proline	118	115
Glycine	330	329
Alanine	129	104
Valine	14	16
Methionine	8.2	11
Isoleucine	6.1	7.8
Leucine	20	26
Tyrosine	2.0	2.2
Phenylalanine	14	15
Hydroxylysine	5.1	23
Lysine	30	13
Histidine	2.0	2.0
Arginine	51	50
Amide nitrogen	(39)	(50)

[a] α1(I) chains are from chick bone collagen (Miller *et al.*, 1967, 1969). [b] α1(II) chains are from third and fourth extracts of sternal cartilages (see text for details).

extracts are of the composition $[\alpha 1(II)]_3$, and that the material chromatographing on CM-cellulose from third and fourth extracts represents α1(II) in essentially pure form.

Amino Acid Analyses of α1(II) and Comparison to α1(I). In order to further characterize the α1(II) chain, amino acid analyses were performed on the material contained in the peak appearing on CM-cellulose chromatograms during chromatography of third and fourth extracts. Amino acid analyses were also performed on fractions from various regions within a given peak and no significant differences could be observed indicating, as suggested above, that the chromatographic heterogeneity of α1(II) does not result from contamination with α1(I). The results of the amino acid analyses on α1(II) are summarized in Table II and compared to similar analyses on α1(I) previously isolated from well-characterized chick bone collagen (Miller *et al.*, 1967, 1969). Comparison of the two types of α1 chains reveals several noteworthy features. α1(II), similar to α1(I), and all vertebrate collagen chains is comprised of one-third glycyl residues. α1(II) and α1(I) have, within experimental error, identical contents of the imino acids and the proportion of hydroxyproline appears to be the same for each chain. Likewise, α1(II) resembles α1(I) in the contents of several other amino acids such as aspartic acid, tyrosine, histidine, and arginine. On the other hand, significant differences between α1(II) and α1(I) are noted in the threonine to serine ratio and, most strikingly, in the contents of glutamic acid, alanine, and leucine. Moreover, although the two types of chain exhibit similar contents of lysine plus hydroxylysine, two-thirds of the lysyl residues are hydroxylated in α1(II) while only 15% of the lysyl residues are hydroxylated in α1(I). Since hydroxylation of lysyl residues occurs after assembly of the primary structure of collagen chains (Udenfriend, 1966), this difference may reflect alterations in the level of lysine hydroxylating enzyme in bone and cartilage or may indicate that a greater proportion of the lysyl residues in α1(II) occur in sequences recognizable as active sites by the enzyme. Aside from these differences between α1(II) and α1(I), there exist some differences in the content of amino acids which are found in relatively small amounts in collagen. From the standpoint of further characterization of α1(II), the difference in methionine content when compared to α1(I) is particularly significant. When the amino acid analysis for α1(I) is expressed in residues of amino acids per chain (using a molecular weight of 95,000 and an average residue molecular weight of 91.2) the α1(I) chain contains 9 methionyl residues giving rise to 10 CNBr peptides which account for all of the molecular weight and amino acids in the chain (Miller *et al.*, 1969). A similar study on α1(II) indicates that this chain contains 12 methionyl residues and characterization of the 13 CNBr peptides derived from the chain after cleavage with CNBr (E. J. Miller, manuscript in preparation) likewise corroborates the analysis of α1(II) listed in Table II.

Carbohydrate Studies. The relatively high hydroxylysine content of α1(II) as well as the development of a distinct yellowish hue during acid hydrolysis of the chain suggested that α1(II) might contain an unusually large amount of protein-bound carbohydrate. This was verified by direct analysis of α1(II) for total protein-bound hexose by the orcinol–sulfuric acid method. The results of these determinations indicated that the hexose content (when calculated in reference to a standard containing galactose and glucose in a 2:1 molar ratio) of α1(II) is approximately 3% with a range of 2.8–3.3%. These values correspond to about 16–18 residues of hexose per chain. In contrast, α1 chains of rat skin collagen (Butler *et al.*, 1967) and the α1(I) chains of chick bone and skin collagen (E. J. Miller, unpublished observations) contain about 0.4% by weight of hexose or 2 residues/chain. Paper chromatography of the carbohydrate of α1(II) after hydrolysis and removal of amino acids by ion-exchange chromatography showed the presence of two components identifiable as glucose and galactose when compared to the migration of known standards. The identification of glucose and galactose as the hexose components of α1(II) suggested that the carbohydrate was linked O glycosidically to the hydroxyl group of hydroxylysine as the disaccharide glucosylgalactose and possibly the monosaccharide galactose as previously determined for several other collagens (Butler and Cunningham, 1966; Spiro, 1969). This point was investigated after periodate oxidation of α1(II). Amino acid analyses of periodate-treated α1(II) demonstrated that 9 of the 23 hydroxylysine residues per 1000 total residues remained and the recovery of hydroxylysine was not diminished by increasing the time of periodate oxidation to 8 and 12 hr. These results, suggesting that the δ-hydroxyl group on approximately 40% of the hydroxylysine residues of α1(II) were substituted by hexose, were confirmed by direct analysis of α1(II) after alkaline hydrolysis. In the system used for amino acid analyses (Miller and Piez, 1966) Glu-Gal-Hylys chromatographs between valine and methionine and Gal-Hylys cochromatographs with tyrosine. Consequently, in determining the Gal-Hylys content of α1(II), a correction factor accounting for the amount of tyrosine in similar samples after acid hydrolysis was employed. Glu-Gal-Hylys and Gal-Hylys exhibited respective color yields of 0.9

TABLE III: The Determination of Hydroxylysine-Linked Carbohydrate in α1(II).

Sample	μmole/mg of α1(II)		
	Hydroxylysine	Glu-Gal-Hylys	Gal-Hylys
Acid hydrolyzed	0.2485 (23.1)[a]		
Periodate treated, acid hydrolyzed	0.1009 (9.2)		
Alkali hydrolyzed	0.1441 (13.3)	0.0585 (5.3)	0.0410 (3.7)

[a] Numbers in parentheses denote residues/1000 residues in total amino acid analysis.

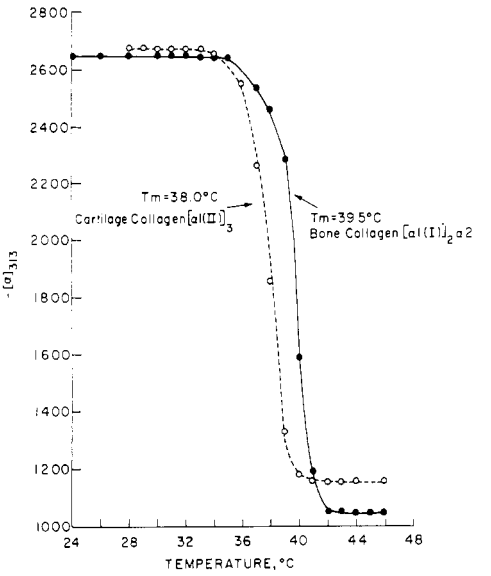

FIGURE 4: Thermal denaturation curves of collagen obtained in third and fourth extracts of sternal cartilages having the chain composition $[\alpha](II)]_3$ and chick bone collagen with the chain composition $[\alpha 1(I)]_2$ 2. The curves were obtained during stepwise increases in temperature of collagen in 0.5 M acetic acid at a concentration of 0.6 mg/ml.

and 0.85 times the value for hydroxylysine. The results on alkaline-hydrolyzed samples showed that approximately 5 of the substituted hydroxylysine residues in α1(II) could be accounted for as Glu-Gal-Hylys and that the remaining substituted residues are present as Gal-Hylys. These results, indicating the presence of approximately 15 residues of hexose per α1(II) chain, are summarized in Table III. They are also in good agreement with the determination of total protein-bound hexose given above.

Polarimetry. The studies cited above have served to differentiate the isolated α1(II) chain from α1(I) by a number of chemical criteria. The collagen to gelatin transition of collagen obtained in third and fourth extracts of sternal cartilages was studied as a means of further characterization at the level of the intact molecule. For comparison, chick bone collagen of the chain composition $[\alpha 1(I)]_2\alpha 2$ (Miller *et al.*, 1967) was similarly studied. The thermal denaturation curves for both types of collagen dissolved in 0.5 M acetic acid, as determined by the change in optical rotation as a function of increasing temperature, are presented in Figure 4. The midpoint between maximal and minimal specific optical rotation was determined to be 39.5° for bone collagen in agreement with previous results (Miller *et al.*, 1967). However, molecules of the chain composition $[\alpha 1(II)]_3$ exhibited a significantly lower melting point of 38.0° with an equally sharp transition. The observed differences in specific optical rotation of the two types of molecules in the native and completely denatured state are not considered significant and probably reflect the experimental error in the determination of collagen concentration by amino acid analyses.

Although these results provide yet another parameter differentiating molecules of the chain composition $[\alpha 1(II)]_3$ from those of the composition $[\alpha 1(I)]_2\alpha 2$, the conditions of pH and ionic strength under which these *in vitro* studies are performed preclude extrapolation of the results with regard to the relative stability of the collagen molecules to *in vivo* conditions.

Discussion

The results cited in this paper are interpreted as indicating that approximately 90% of the extractable collagen from cartilage is comprised of the α1(II) chain. This figure may be calculated from an examination of the chromatographic properties of the collagen obtained in each extract, estimates of the proportion of α1(II) in the α1 fraction of each extract, and the total amount of collagen brought into solution during each extraction period. With reference to Table I, the results show that approximately 80% of the collagen in first extracts is α1(II), 95% in second extracts is α1(II), and all the collagen in subsequent extracts is α1(II). When these results are considered in terms of the amount of collagen in each extract, the proportion of α1(II) in the extractable collagen is about 90%. These results for the proportion of α1(II) in the extractable cartilage collagen of lathyritic chicks are in good agreement with previous estimates of the proportion of α1(II) in the total mass of insoluble collagen in the cartilages of control animals (Miller and Matukas, 1970).

The question with regard to the distribution of α1(I), α1(II), and α2 in triple-stranded molecules brought into solution during the first extraction period cannot be resolved with certainty at this time. However, the extraction of molecules of the chain composition $[\alpha 1(II)]_3$ in later extracts would suggest that the collagen in first extracts is a mixture of molecules with the chain compositions $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$. Indeed, the proportion of α1(II) in the first extract of finely divided cartilages where the chain ratio is approximately $8\alpha 1(II):2\alpha 1(I):1\alpha 2$ indicates that at least some of the α1(II) chains would of necessity be the exclusive components of a triple-stranded structure.

Utilizing successive extraction periods for the solubilization of cartilage collagen along with the apparent localization of molecules of the chain composition $[\alpha 1(I)]_2\alpha 2$ at the periphery of the tissue conveniently afforded the opportunity to extract only molecules of the chain composition $[\alpha 1(II)]_3$ in later extracts. Amino acid analyses of the α1(II) chain isolated in pure form from later extracts revealed that α1(II) differs substantially from α1(I) in the content of several amino acids. These results at the level of the whole chain support the previous conclusion (Miller and Matukas, 1969) based on the amino acid differences in homologous peptides α1(II)-CB2 and α1(I)-CB2, that synthesis of α1(II) is directed by a

different structural gene from that responsible for the synthesis of α1(I).

Amino acid analyses also showed that α1(II) could be further differentiated from α1(I) in that almost two-thirds of its lysyl residues are hydroxylated. In this regard, the amino acid analysis of α1(II) bears a close resemblance to that of whole glomerular basement membrane (Kefalides and Winzler, 1966; Spiro, 1967a) and lens capsule (Fukushi and Spiro, 1969; Denduchis *et al.*, 1970) as well as the soluble collagen obtained from these structures after limited digestion with proteolytic enzymes (Kefalides, 1968; Kefalides and Denduchis, 1969). In addition, similar to glomerular basement membrane collagen (Spiro, 1967b,c) and lens capsule (Spiro and Fukushi, 1969), α1(II) exhibits a high degree of glycosylation of the hydroxylysine residues. However, the extent of glycosylation of hydroxylysine residues observed in this study for α1(II) is 40% which is approximately one-half that observed for basement membrane and lens capsule collagens, and the carbohydrate appears to be almost equally distributed between disaccharide glucosylgalactose and monosaccharide galactose in α1(II) whereas essentially all of the hydroxylysine-linked carbohydrate is in the disaccharide form in membrane collagens. Nevertheless, it is to be expected that postsynthetic modifications of this type would be highly dependent on the age and condition of the animals under study and it is impossible to state at present whether or not the carbohydrate content of α1(II) observed in this study represents a maximum value. Of central importance in these considerations is the observation that α1(II) from chick sternal cartilages contains approximately nine times as much hydroxylysine-linked carbohydrate as α1(I) from bone and skin collagen. It seems reasonable to speculate that extensive glycosylation of hydroxylysine residues in cartilage collagen may occur in order to promote collagen–proteoglycan interaction through the carbohydrate side chains of both types of macromolecule. Although little information on this point is available, such interactions could be of major significance in maintaining the structural integrity and properties of cartilaginous tissues.

An additional finding worthy of comment is the observation that molecules of the composition $[\alpha 1(II)]_3$ are somewhat less heat stable in acid solution than those of the composition $[\alpha 1(I)]_2\alpha 2$. Since both types of collagen molecules used in this study were obtained from the same species and since the analyses of α1(I) and α1(II) presented above along with those for the α2 chain (Miller *et al.*, 1967; Lane and Miller, 1969) indicate that both types of molecule have identical imino acid contents, these results are apparently at variance with the known correlations of body temperature, imino acid content, and collagen stability (Harrington and von Hippel, 1961). At present, no definitive explanation for this apparent inconsistency can be offered; nevertheless, the decreased stability of molecules of the chain composition $[\alpha 1(II)]_3$ may reflect the absence of additional minor stabilizing forces such as charge–charge interactions during the unfolding of three identical chains as opposed to the situation where one of the chains differs from the other two. In addition, it is possible that the carbohydrate moieties associated with α1(II) render molecules comprised of the chain less stable although the manner in which this influence might be exerted is, at present, entirely unknown.

The results presented in this paper on sternal cartilage collagen combined with similar results on the epiphyseal growth plates of embryonic chick bones (Miller and Matukas, 1970) indicate that replacement of cartilage by bone during development involves the regulation and control of collagen synthesis in the sense that collagen synthesis proceeds from different genetic loci in the two tissues. Failure to correctly regulate collagen synthesis in these tissues could be responsible for a number of developmental anomalies occurring during embryogenesis as well as several connective tissue disorders which are manifest in the adult.

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References

Bornstein, P. (1969), *Biochemistry 8*, 63.
Bornstein, P., and Piez, K. A. (1964), *J. Clin. Invest. 43*, 1813.
Butler, W. T., and Cunningham, L. W. (1966), *J. Biol. Chem. 241*, 3882.
Butler, W. T., Piez, K. A., and Bornstein, P. (1967), *Biochemistry 6*, 3771.
Denduchis, B., Kefalides, N. A., and Bezkorovainy, A. (1970), *Arch. Biochem. Biophys. 138*, 582.
Fukushi, S., and Spiro, R. G. (1969), *J. Biol. Chem. 244*, 2041.
Harrington, W. F., and von Hippel, P. H. (1961), *Advan. Protein Chem. 16*, 1.
Kang, A. H., Igarashi, S., and Gross, J. (1969b), *Biochemistry 8*, 3200.
Kang, A. H., Piez, K. A., and Gross, J. (1969a), *Biochemistry 8*, 1506.
Kang, A. H., Piez, K. A., and Gross, J. (1969c), *Biochemistry 8*, 3648.
Kefalides, N. A. (1968), *Biochemistry 7*, 3103.
Kefalides, N. A., and Denduchis, B. (1969), *Biochemistry 8*, 4613.
Kefalides, N. A., and Winzler, R. J. (1966), *Biochemistry 5*, 702.
Lane, J. M., and Miller, E. J. (1969), *Biochemistry 8*, 234.
Lewis, M. S., and Piez, K. A. (1964), *J. Biol. Chem. 239*, 3336.
Miller, E. J. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 1839.
Miller, E. J., Lane, J. M., and Piez, K. A. (1969), *Biochemistry 8*, 30.
Miller, E. J., Martin, G. R., Piez, K. A., and Powers, M. J. (1967), *J. Biol. Chem. 242*, 5481.
Miller, E. J., and Matukas, V. J. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1264.
Miller, E. J., and Matukas, V. J. (1970), *in* Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 1, Balazs, E. A., Ed., New York, N. Y., Academic Press, p 109.
Miller, E. J., and Piez, K. A. (1966), *Anal. Biochem. 16*, 320.
Nordwig, A., and Hayduk, U. (1969), *J. Mol. Biol. 44*, 161.
Piez, K. A. (1965), *Biochemistry 4*, 2590.
Piez, K. A. (1968), *Anal. Biochem. 26*, 305.
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Piez, K. A., Martin, G. R., Kang, A. H., and Bornstein, P. (1966), *Biochemistry 5*, 3813.
Pikkarainen, J. (1968), *Acta Physiol. Scand. Suppl.*, 309.
Pikkarainen, J., Rantanen, J., Vastamaki, M., Lampiaho, K., Kari, A., and Kolonen, E. (1968), *Eur. J. Biochem. 4*, 555.
Spiro, R. G. (1967a), *J. Biol. Chem. 242*, 1915.
Spiro, R. G. (1967b), *J. Biol. Chem. 242*, 1923.
Spiro, R. G. (1967c), *J. Biol. Chem. 242*, 4813.

Spiro, R. G. (1969), *J. Biol. Chem. 244*, 602.
Spiro, R. G., and Fukushi, S. (1969), *J. Biol. Chem. 244*, 2049.
Tsiganos, C. P., and Muir, H. (1969), *Biochem. J. 113*, 879.
Udenfriend, S. (1966), *Science 152*, 1335.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 279.

Implication of Tyrosine in Iron Binding in Hemerythrin*

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ABSTRACT: In a study of the nitration of hemerythrin by tetranitromethane it has been found that in the apoprotein all five possible tyrosines react, but in oxyhemerythrin and in methemerythrin monomer only three residues react. Pepsin hydrolysis of nitrated oxyhemerythrin yields two yellow spots on peptide mapping. The slower moving spot contains 2 moles of nitrotyrosine arising from tyrosine 18 and 70. The faster moving spot is a peptide containing nitrotyrosine 67. The results suggest unreacted tyrosines 8 and 109 are each chelated to iron. There was no differential reactivity toward *N*-bromosuccinimide oxidation, suggesting tryptophan is not a ligand to iron.

We have recently shown that four of the seven histidine residues of the oxygen carrying nonheme iron protein, hemerythrin, serve as ligands to iron and may constitute a portion of the active site, Fan and York (1969). Assuming octahedral coordination and reserving a coordination position for oxygen which could be alternately occupied by water leave three ligands per iron atom unaccounted for. Besides the histidine just noted and the cysteine which has been ruled out, Keresztes-Nagy and Klotz (1965), the next most likely group to fill the remaining ligand positions is tyrosine.

The selectivity of tetranitromethane (TNM) for tyrosine and sulfhydryl groups under mild conditions has been demonstrated, Riordan *et al.* (1966, 1967). Also, it has been shown that iron protects coordinated tyrosine residues in transferrin against nitration by TNM, Line *et al.* (1967). These characteristics of TNM reactivity suggested to us that those residues of tyrosine in hemerythrin which were coordinated with iron would not be nitrated by TNM whereas those tyrosines not coordinated would react with TNM to form the yellow 3-nitrotyrosine. After pepsin hydrolysis those peptides containing the nitrotyrosine would be easily identifiable. Knowing specifically which residues did react would allow us to infer which were coordinated with the iron or buried and unavailable to the reagent. A preliminary account of this work has appeared, York and Fan (1970). It has been recently reported that TNM reacts with all five tyrosine residues in methemerythrin azide, pH 8, Rill and Klotz (1970). Under their experimental conditions these authors were not able to obtain any information as to the involvement of a specific tyrosine residue in the iron binding site although there was a qualitative implication of tyrosine as a ligand. Klippenstein *et al.* (1968) have published the amino acid sequence of hemerythrin which makes this study possible.

Materials and Methods

Preparation of Hemerythrin and Derivatives. Oxy- and methemerythrin were prepared as previously described, Fan and York (1969). Methemerythrin monomers were prepared by reaction with cyanogen bromide, Keresztes-Nagy and Klotz (1965). Heat-denatured hemerythrin which was soluble at pH 7–8 was prepared by heating a solution of oxyhemerythrin (2–10 mg per ml made up in 0.1 M phosphate at pH 8.0, 0.1% sodium lauryl sulfate, and 10^{-3} M in EDTA) at 50° until colorless. The solution was then dialyzed against the Tris–sodium lauryl sulfate buffer to remove the iron and EDTA.

Reaction with Tetranitromethane. The number of TNM reactive tyrosine residues in various forms of hemerythrin was determined in the following way. A working TNM (Aldrich) solution was prepared by saturating a 50% aqueous ethanol solution at room temperature. The concentration of TNM in this solution was determined by the amount of nitroformate, ϵ_{350} 14,000 M^{-1} cm^{-1}, Sokolovsky *et al.* (1969), released upon treating an aliquot with a large excess of cysteine at pH 7.0 in 0.1 M Tris chloride. At 27° this value was determined to be 25 μmoles of TNM/ml. All experiments were performed at a final concentration of 1 mg/ml of protein, 5–7% ethanol, and a 50-fold molar excess of TNM in 0.1 M Tris-chloride, pH 8.3. Complete reaction required 18 hr at 0°. After this time no further nitration of tyrosine in oxyhemerythrin occurred even with addition of fresh TNM. In order to remove nitroformate which interferes with the spectroscopic assay, the reaction mixture was dialyzed against 0.1 M Tris, pH 8.0, then precipitated with perchloric acid, redissolved in 0.1 M KOH, and dialyzed against 0.1 M Tris, pH 8.8, and the optical density was determined at 428 nm. The concentration of 3-nitrotyrosine was determined using ϵ_{428} 4100 M^{-1} cm^{-1}, Riordan *et al.* (1966). In order to study the kinetics, the reaction was stopped at a given time by the addition of excess cysteine which rapidly removed the excess TNM by oxidation of the cysteine,

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