Characterization of Chick Bone Collagen and Compositional Changes Associated with Maturation

(Received for publication, June 21, 1967)

E. J. MILLER, G. R. MARTIN, K. A. PIEZ, AND M. J. POWERS

From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Collagen extracted from the diaphyseal region of the tibiae of normal and lathyritic chicks with 0.5 M acetic acid and 5 M guanidine hydrochloride at neutral pH has been studied with regard to chain organization and amino acid composition. Decalcification by dialysis against large quantities of 0.5 M acetic acid and subsequent exposure of the decalcified bones to fresh 0.5 M acetic acid followed by 5 M guanidine resulted in the solubilization of 85% of the total collagen in the bones from lathyritic animals. Collagen in the bones of control animals was almost entirely resistant to extraction with 0.5 M acetic acid, and less than 20% of the total collagen could be extracted in 5 M guanidine. These observations suggest a high degree of intermolecular cross-linking. Chromatography of the collagen extracted by both solvents from the bones of lathyritic animals on columns of carboxymethyl cellulose indicated that the extracts were composed almost entirely of single- and double-chain components. Carboxymethyl cellulose chromatography of the collagen extracted with 5 M guanidine from the bones of normal chicks revealed that only 30% of the dissolved collagen (5% of the total collagen) could be recovered as these components. Chromatography on an agarose molecular sieve column showed that the remainder could be accounted for in part by high molecular weight aggregates and in part by low molecular weight, degraded material. These results suggest that bone collagen fibrils are stabilized through the formation of intra- and intermolecular cross-links and that lathyrogens exert their effects by inhibiting cross-link formation, as previously shown for soft tissue collagens. The extractability of normal bone collagen in guanidine appears to result from degradation in the presence of the denaturing agent to yield a highly heterogeneous mixture.

Carboxymethyl cellulose chromatography of acid-extracted collagen from the bones of lathyritic animals indicated that the bone collagen molecule contains two chains (α1) that are identical or similar and one chain (α2) that is unlike the other two. Their compositions are analogous to those of other vertebrate collagens.

Amino acid analyses of the chromatographically separated α chains in successive extracts of collagen from the lathyritic bones indicated that during development there was a progressive decrease in hydroxylysine content, accompanied by an equivalent increase in lysine content, with no significant change in the content of any other amino acid. This observation was verified by amino acid analyses of the total bone collagen of normal chicks of various ages. The hydroxylysine content decreased by nearly one-half, from 11 to 6 residues per 1000, between the 1st and the 49th day after hatching. This result suggests that the hydroxylation of bone collagen lysyl residues is dependent on various factors extrinsic to the collagen molecule.

X-ray diffraction studies (1) indicate that the collagen molecule has a coiled coil, triple chain structure. Further information about the structure of various vertebrate collagens has been derived by examining the components produced when soluble collagens are denatured. The components obtained from salt-extracted collagen are predominantly of molecular weight of about 95,000, and are termed α components or α chains (2–5). In most collagens so far examined the amino acid composition of one of the three α chains (the α2 chain) differs significantly from that of the other two (the α1 chains), making possible the separation of the two types by chromatography on carboxymethyl cellulose (4–6). All three α chains of codfish skin collagen, however, differ in amino acid composition and can be separated by CM-cellulose chromatography (7, 8). Other components of higher molecular weight than the α chains have been found in significant amounts in solutions of denatured, acid-extracted collagens. Covalently cross-linked components containing two α chains are called β components (2–4), and those with three α chains have been designated γ components (9–11). These high molecular weight components have been shown to arise by intramolecular cross-linking of α chains (4, 12–14).

The presence of cross-linked components of soft tissue collagens coincides with a decrease in the case of extraction of collagen from the tissue. Collagen that is not highly cross-linked or collagen with largely intramolecular cross-links can be extracted from soft tissues with solvents such as cold salt (NaCl) solutions.
at neutral pH and dilute acetic acid (4), while varying amounts of more highly cross-linked collagen can be removed by extraction with denaturing solutions, such as 5 M guanidine hydrochloride (6, 15). In the latter solvent, the structure of the native collagen molecule is destroyed (16). Depending on the species, the tissue, and the age of the animal, a considerable amount of collagen remains insoluble even after extraction with 5 M guanidine-HCl.1

Since bone collagen is largely insoluble in the solvents that are used to extract native collagen, it has been more difficult to characterize. In this paper, we report our studies on the characterization of collagen extracted with 0.5 M acetic acid and 5 M guanidine-HCl from the tibiae of chicks fed β-amino propionitrile fumarate, a lathyrinogen that inhibits cross-linking of collagen and renders it more readily extractable (12, 13, 17). In addition, we have studied the molecular organization of collagen extracted with 5 M guanidine-HCl from the tibiae of normal chicks. In several important respects our results differ from those of Glimcher and Katz (18), Glimcher, Katz, and Travis (19), Glimcher et al. (20), and Francois and Glimcher (21), who have also studied chick bone collagen.

EXPERIMENTAL PROCEDURE

Source of Collagen—White Leghorn chicks, obtained from a local hatchery at the age of 1 day, were maintained on a commercial ration for various intervals as required by the experimental procedure. Lathyrism was induced by feeding the chicks a commercial diet to which was added 1 g of β-amino propionitril fumarate per kg from the time of arrival until 3 weeks of age. At this time, the control and lathyritic animals (120 in each group) were killed by decapitation, and the tibiae were immediately removed and rinsed several times in cold (5°) 0.16 M NaCl and then in water. After the epiphyseal ends had been carefully removed in order to preclude contamination with cartilaginous areas, the bones were cleaned of periosteum and endosteal tissue. The remaining tissue, consisting of long thin cylinders of diaphyseal bone, was freeze-dried and weighed. The intact bone cylinders were used for all subsequent procedures.

Calculation of Bone Collagen and Mineral Content—In each experiment 50 dry bone cylinders from each group were decalcified by constant stirring in 500 ml of cold 0.35 M EDTA, as the potassium salt, at pH 8.1. Decalcification, as judged by the absence of an ash residue when the bones were heated at 600° for 24 hours in platinum crucibles, was complete within 4 days at this temperature. The bones from both control and lathyrinic animals became somewhat turbid and viscous, while the solution in contact with the bones from control animals remained clear with little apparent increase in viscosity. The solution remaining in the dialysis bags after decalcification by dialysis against 0.5 M acetic acid will subsequently be referred to as the first acid extract. The residues remaining after decalcification were extracted for 1 week with constant stirring in 200 ml of fresh 0.5 M acetic acid. The collagen solubilized during this extraction procedure was termed the second acid extract. An additional extraction period of 1 week yielded only a small amount of additional collagen.

Following the third acid extraction the bone residues were extracted for 1 week with constant stirring in 200 ml of 5 M guanidine-HCl (clarified by filtration through activated charcoal) at pH 7.0. Extraction of the residues for additional weekly periods resulted in the solubilization of only small amounts of collagen. The residues remaining after guanidine-HCl extraction were thoroughly washed with distilled water to eliminate all of the guanidine, and were dried by lyophilization.

All extracts were clarified by centrifugation at 100,000 x g for 2 hours in a Spincgo model L ultracentrifuge. Collagen contained in the 0.5 M acetic acid extracts was precipitated by dialysis against large volumes of 0.02 M disodium phosphate. The precipitate was collected by centrifugation at 40,000 x g for 30 min, redissolved in 0.5 M acetic acid, and precipitated again by the addition of sufficient sodium chloride to give a concentration of 5%. The precipitate was collected by centrifugation, redissolved in 0.5 M acetic acid, dialyzed against a large volume of the same solvent, and lyophilized. No attempt was made to further purify the collagen extracted by 5 M guanidine-HCl, since amino acid analyses of this material indicated that collagen comprised approximately 98% of the total protein (Table III, below). Therefore, an aliquot of the guanidine-HCl extracts was dialyzed exhaustively against water, lyophilized, and weighed to determine the approximate collagen concentration. The remainder was dialyzed directly against a large volume of 0.06 M 1/2 sodium acetate buffer, pH 4.8, in preparation for CM-cellulose chromatography.

CM-cellulose Chromatography—A weighed sample of acetic acid-extracted collagen (15 to 25 mg) was suspended in 20 ml of starting buffer, 0.06 M 1/2 sodium acetate, pH 4.8, and stirred for a few hours at 5° until the collagen was completely dissolved. The solutions were warmed to 45° for 30 min and applied by means of a peristaltic pump (LKB ReCyChrom) to a column, 15 x 75 mm, of CM-cellulose (Whatman, microgranular CM-32, capacity, 1.0 meq per g) that had previously been equilibrated with starting buffer. The column temperature was maintained at 42°. Fractionation of the material applied to the column was achieved essentially as described by Piez, Eigner, and Lewis (4). A linear gradient was obtained by means of a two-chamber constant level device containing 200 ml of starting buffer in the first chamber and 200 ml of limit buffer (0.06 M 1/2 sodium acetate containing 0.1 M NaCl, pH 4.8) in the second chamber. The column was operated at a flow rate of 150 ml per hour. Chromatography of the collagen contained in the 5 M guanidine-HCl extracts was

1 The abbreviation used is: guanidine-HCl, guanidine hydrochloride.
performed in the same manner, except that the samples were clarified by filtration through medium porosity sintered glass
disks after being warmed in the starting buffer.

The column effluent was monitored continuously at 230 μm
by the use of a flow cell (10-mm light path, 0.3-ml volume) in a
Beckman DB spectrophotometer, and absorption was recorded by
a Sargent SRL strip chart recorder equipped with logarithmic
gears and operated at a speed of 6 inches per hour. The column
effluent was collected in 10-ml fractions. Combined fractions

The area under the peaks in each chromatogram was calculated
by planimetry. A conversion factor for determining sample
weights from areas under the peaks was obtained by chromato-
graphing a well characterized sample of rat skin collagen that was
known to be 100% recoverable as α and β components. The
percentage of total sample collagen recovered as various com-
ponents after CM-cellulose chromatography could then be de-
temined.

**Molecular Sieve Chromatography**—Samples containing about
100 mg of extracted collagen were dissolved in 10 ml of 1 M CaCl₂
buffered with Tris (0.05 M, pH 7.5) by warming to 40° for 15 to
30 min and were placed on a column, 2.5 × 110 cm (540 ml),
of Sepharose 4B (Pharmacia) at room temperature. The column
was equilibrated and eluted with the same solvent at 40 ml per
hour. The effluent was monitored as described for CM-cellulose
chromatography.

**Polarimetry**—Collagen obtained in the first acid extract of
bones from lathyritic animals was dissolved in either 0.5 M
acetic acid or 0.15 M potassium acetate, pH 4.8, at a concentra-
tion of 1 mg per ml. Optical rotation of the samples in both
solvents was measured at 313 μm in a Rudolph model 80 pho-
to-electric spectropolarimeter with a 1 dm jacketed cell and with a
mercury arc lamp as the light source. Denaturation curves were
obtained by a stepwise increase in temperature over the range,
22–50°. At each temperature, 30 min were allowed for equilibra-
tion. Collagen concentrations in the solutions used for polar-
imetry were calculated from the nitrogen concentration (micro-
Kjeldahl), assuming a nitrogen content of 18.6% for collagen.

**Acrlylamide Gel Electrophoresis**—Disc electrophoresis of the
fractions obtained during CM-cellulose chromatography of
collagen extracted with 0.5 M acetic acid and 5 M guanidine-HCl
was performed according to the procedure of Nagai, Gross, and
Piez (22).

**Amino Acid Analyses**—Samples of bone collagen (2 to 5 mg)
were hydrolyzed in sealed tubes under nitrogen in 3 ml of constant
boiling 6 N HCl for 24 hours at 108°. Amino acid analyses of the
hydrolysates were performed on an automatic amino acid ana-
lyzer (23) as modified for accelerated analyses (24). In the final
calculations, corrections were made for the loss of labile amino
acids (threonine, serine, tyrosine, and methionine) and for in-
complete release of valine (25).

**RESULTS**

In Table I are listed data on the relative amounts of mineral
and organic phases present in the bones used in these studies.
It is seen that lathyrism caused little alteration in the relative
proportion of the mineral and organic phases in the diaphyseal
region of chick tibiae. These results are in agreement with those
of Glimcher et al. (20), who studied the mineral and organic
phases of metatarsal and tibial bones in normal and lathyritic
chick embryos. The decrease in total mass observed for lath-
rytic chick bone is compatible with the 10 to 15% decrease in
total body weight observed in the experimental animals. The
decrease in body weight was most probably the result of an in-
sufficient dietary intake due to immobilization during the last
few days of β-aminopropionitrile feeding. Although small
amounts (0.5 to 1.0 mg per bone) of protein were removed from
the bones during decalcification in the EDTA solutions, amino
acid analysis of this material indicated that no collagen had been
solubilized. Amino acid analysis also showed that collagen ac-
counted for 98% of the protein of the organic residues following
decalciﬁcation. These results for avian bones are similar to
those obtained for mammalian compact bone by Eastoe and
Eastoe (28), who found that collagen accounted for 94% of the
total organic matter of ox femur diaphyses.

The extractability of the bone collagen in the solvents used is
given in Table II. The results were calculated from the amount
of collagen obtained in the individual extracts and are expressed
as a percentage of the total collagen contained in the calcified
starting material, assuming collagen to be 23% of the dry weight
of the calcified bones. During decalcification and extraction
with 0.5 M acetic acid, less than 1% of the total collagen in bones
from control chicks was solubilized. Even 5 M guanidine-HCl
was relatively ineffective in removing collagen from this tissue.
In contrast, appreciable quantities of collagen in the bones from
lathyritic animals were solubilized in acetic acid, and guanidine-
HCl dissolved the majority of the rest.

The markedly increased extractability of collagen from the 3-
week-old lathyritic chick bone probably resulted from the fact
that this collagen was synthesized for the most part in the pres-
ence of the lathyrugen. The average weight of the dry decalci-
fied diaphyseal region of the tibia of the newly hatched chick is 3.8

---

**Table I**

Analysis of diaphyseal region of control and lathyritic chick tibiae
for mineral and organic content

<table>
<thead>
<tr>
<th>Bone</th>
<th>Weight</th>
<th>Mineral content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/bone, dry wt</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>74.0</td>
<td>77.0</td>
</tr>
<tr>
<td>Lathyritic</td>
<td>63.2</td>
<td>77.6</td>
</tr>
</tbody>
</table>

**Table II**

Percentage of total bone collagen extracted by 0.5 M acetic acid and
5 M guanidine hydrochloride

The values given are averages of data obtained in two sepa-
rate experiments.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Control</th>
<th>Lathyritic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M acetic acid</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>First extract (decalcification)</td>
<td>&lt;1</td>
<td>13</td>
</tr>
<tr>
<td>Second extract</td>
<td>17</td>
<td>64</td>
</tr>
</tbody>
</table>

---

Issue of December 10, 1967 Miller, Martin, Piez, and Powers
mg. As seen in Table I, this value increases to 14.2 mg at 3 weeks of age. Moreover, remodeling during growth would lead to the removal of all or most of the normal bone collagen present when feeding of the lathyrogen was begun.

A chromatogram obtained by CM-cellulose chromatography of the collagen present in the first acetic acid extract of bones from lathyritic animals is shown in Fig. 1. The peaks were identified as \( \alpha \) or \( \beta \) components by their migration during gel electrophoresis. Amino acid analyses established that the peak labeled \( \beta_{12} \) was composed of an equal mixture of \( \alpha_1 \) and \( \alpha_2 \) chains (Table III). Protein in fractions taken across the \( \alpha_1 \) peak (Fractions I to V, Fig. 1) did not vary significantly in amino acid composition. This chromatogram resembles previously published chromatograms of soft tissue collagens, such as rat skin and tail tendon (4), shark skin (5), and human skin (6) collagens, in that it contains three prominent components, \( \alpha_1 \), \( \alpha_2 \), and \( \beta_{12} \). The \( \beta_{11} \) component frequently observed in chromatograms of other collagens was not present in sufficient amounts to be readily apparent. However, material with the mobility of a \( \beta \) component was observed by acrylamide gel electrophoresis in the trailing edge of the \( \alpha_1 \) peak when more highly cross-linked bone collagen was chromatographed (see below). The ratio of the area of \( \alpha_1 \) to that of the \( \alpha_2 \) component was 2:1, consistent with the presence of two \( \alpha_1 \) and one \( \alpha_2 \) chains per molecule, as found for other collagens (4). Calculation of the area under the peaks of the chromatogram showed that within experimental error all of the collagen in the first acid extract was recovered as \( \alpha_1 \), \( \beta_{11} \), and \( \alpha_2 \) components.

A chromatogram of the collagen obtained in the second acid extract of bones from lathyritic animals is presented in Fig. 2. The elution pattern resembles that observed for collagen in the first acid extract, although the double-chain \( \beta_{12} \) component comprises a somewhat greater proportion of the total collagen. Of the collagen in the sample, 100% was accounted for as the observed components.

In Fig. 3 is presented the chromatogram of collagen extracted from the bones of lathyritic animals with 5 M guanidine-HCl. The forepeak consisted of a strongly absorbing noncollagenous contaminant of the preparation which accounted for only a small percentage of the weight of the sample. Hydrolysate of this material contained hexosamines and no hydroxyproline. Broadening of the peaks, indicative of heterogeneity, was apparent. As shown below, this resulted from the lengthy exposure to the denaturing solvent as well as from the presence of unresolved high molecular weight components. A part of the

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Elution pattern of 14 mg of denatured lathyritic chick bone collagen (first acid extract) chromatographed on CM-cellulose with a linear gradient of ionic strength from 0.06 to 0.16 at pH 4.8. Protein from Fractions I to VII was taken for amino acid analyses and gel electrophoresis.

**Table III**

Amino acid composition of 3-week-old chick bone collagen

The values given represent averages of duplicate analyses.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Lathyritic bone</th>
<th>Control bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \alpha_1 )</td>
<td>( \beta_{11} )</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>42</td>
<td>46</td>
</tr>
<tr>
<td>Threonine</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Serine</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>Proline</td>
<td>118</td>
<td>118</td>
</tr>
<tr>
<td>Glycine</td>
<td>329</td>
<td>329</td>
</tr>
<tr>
<td>Alanine</td>
<td>129</td>
<td>129</td>
</tr>
<tr>
<td>Valine</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Methionine</td>
<td>8.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.1</td>
<td>11</td>
</tr>
<tr>
<td>Leucine</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>Amide nitrogen*</td>
<td>(39)</td>
<td>(45)</td>
</tr>
</tbody>
</table>

\* The collagen was that obtained in the first 0.5 M acetic acid extraction.

\* The collagen was that obtained by extraction with 5 M guanidine hydrochloride. \( \beta_{11} \) had the same composition as \( \alpha_1 \).

\* Not included in calculation of total residues.
material chromatographing with $\beta_{12}$ in Region III failed to enter the separating gel during the disc electrophoresis, indicating the presence of material at least as large as the $\gamma$ component. A part of the material in Region II (Fig. 3) displayed the mobility of a $\beta$ component, and is listed as $\beta_{11}$ since this region had the same amino acid composition as Region I. The $\beta$ components comprised a greater proportion of the collagen than in the second acid extract. Planimetry of the area under the peaks on three chromatograms of guanidine-extracted collagen from bones of lathyritic animals indicated that about 99% of the extracted collagen was recovered under the conditions used.

The collagen in the bones from normal animals that could be extracted by 0.5 M acetic acid was insufficient to allow purification and fractionation by CM-cellulose chromatography. A representative chromatogram of the collagen extracted from control bones by 5 M guanidine-HCl is shown in Fig. 4. The elution pattern showed the expected components but again indicated marked heterogeneity, similar to the results for collagen extracted from bones of lathyritic animals by guanidine-HCl (Fig. 3). The forepeak was found to represent a noncollagenous contaminant, similar to that seen in guanidine-HCl extracts of bones from lathyritic animals, although it was relatively larger, perhaps because the proportion of collagen solubilized was smaller.

Gel electrophoresis of material chromatographing with $\beta_{12}$ in Region III likewise indicated the presence of unresolved high molecular weight components, and $\beta_{11}$ was clearly observed in Region II (Fig. 4). The amino acid compositions of the total guanidine-HCl extract and the $\alpha_1$, $\alpha_2$, and $\beta$ components of the collagen extracted by guanidine-HCl from control bones are listed in Table III. The components obtained from guanidine-extracted collagen and acid-extracted lathyritic collagen do not differ significantly in composition. The amino acid composition of the total extract from control bone indicates the presence of a small percentage of contaminating protein.

Calculations of the area under the peaks observed in chromatograms of collagen from control bones showed that only about 30% of the collagen originally extracted by 5 M guanidine-HCl was actually recovered. In contrast to collagen extracted by guanidine-HCl from lathyritic bones, some of the guanidine-extracted collagen from control bones would not dissolve on warming in the starting buffer and therefore could not be applied to the column. This fraction, presumably highly cross-linked material, and the soluble high molecular weight components that are not eluted from the column under the conditions used account in part for the low recoveries (see below). Similar high molecular weight aggregates have been studied by Veis and Ansewy (14), who used elution at high salt concentrations and buffers containing urea.

These results, expressed as recovery of collagen from normal and lathyritic bones as $\alpha$ and $\beta$ components, are summarized in Table IV. Only 5% of the total collagen in normal bone was recovered, while about 78% was recovered from lathyritic bone. If only the single-chain $\alpha_1$ and $\alpha_2$ components are considered, these recoveries become 4% and 59% of total bone collagen, respectively.

A possible factor contributing to the broadened peaks seen in CM-cellulose chromatography of guanidine-extracted collagen was thought to be degradation occurring in the denaturing

**TABLE IV**

<table>
<thead>
<tr>
<th>Bones and extraction solvent</th>
<th>Component</th>
<th>Total recovery</th>
<th>Total as percentage of total bone collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$</td>
<td>$\alpha_2$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Normal*</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>5 M guanidine</td>
<td>17b</td>
<td>5</td>
<td>8c</td>
</tr>
<tr>
<td>Lathyritic</td>
<td>62b</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>First 0.5 M acetic acid</td>
<td>57b</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>Second 0.5 M acetic acid</td>
<td>40b</td>
<td>23</td>
<td>27c</td>
</tr>
<tr>
<td>5 M guanidine</td>
<td>62b</td>
<td>31</td>
<td>7</td>
</tr>
</tbody>
</table>

* Less than 1% of the collagen in normal bones could be extracted by 0.5 M acetic acid (Table II).

b Includes $\beta_{11}$; see text.

c Includes some $\gamma$ component; see text.
solvent. To test this hypothesis, acid-extracted lathyritic collagen was dissolved in 5 M guanidine-HCl, allowed to stand for 1 week at 5°C, dialyzed against strating buffer, and then chromatographed on CM-cellulose. As illustrated in Fig. 5 (compare with Fig. 2), this treatment caused a marked broadening of the peaks, indicative of heterogeneity. Recoveries were about 70%.

To characterize further the guanidine-extracted collagen from normal bone, samples were chromatographed on 4% agarose beads (Pharmacia 4B, Pharmacia), a molecular sieve material with a fractionation range between about 30,000 and 2 x 10^6. The collagens were kept in the denatured state at room temperature by the use of 1 M CaCl_2 as the solvent. Fig. 6 shows a chromatogram of guanidine-extracted collagen from normal chick bone compared to a chromatogram of acid-extracted collagen from lathyritic chick bone. As in the case of CM-cellulose chromatography (Fig. 1), the lathyritic collagen could be entirely accounted for as α and β components. However, the guanidine-extracted normal collagen showed extreme heterogeneity, with molecular weights ranging from the millions to values considerably below the 95,000 expected for α chains. A major peak in the position of α chains (but considerably broadened), a smaller amount of β component, and a peak that may represent γ component were apparent. Recoveries were about 80% as measured by weighing the protein after dialysis and lyophilization of the effluent fractions. It is clear that the very low recoveries after CM-cellulose chromatography must be accounted for not only by high molecular weight aggregates but also by low molecular weight degraded products. The latter may appear throughout the effluent from CM-cellulose columns and may not be apparent as discrete peaks. This would result in an elevated base-line, which would be difficult to detect and would not be included in the measurement of peak areas.

Amino acid analysis and the weight of protein in the fractions from the agarose column indicated that the absorbance was a reasonably good measure of collagen concentration. The noncollagenous, ultraviolet light-absorbing protein that appeared as a forepeak in the effluents from CM-cellulose columns was largely confined to the excluded peak and the tail of the low molecular weight material in the effluent from the agarose column. Its presence in these regions was indicated by measurable amounts of hexosamine and slightly low values for glycine and hydroxyproline in hydrolysates of the proteins.

To rule out the possibility that the difficulty in redissolving guanidine-extracted collagen from normal bone and the poor recoveries from CM-cellulose columns might be explained in part by reaggregation of the denatured collagen, protein from various portions of the effluent from the agarose column was isolated by dialysis against water, lyophilized, and rechromatographed on the same column. In each case the fraction redissolved readily in 1 M CaCl_2 and chromatographed in the original position with, however, a pronounced shifting of the protein toward lower molecular weights, again indicative of degradation. Recoveries were complete within experimental error, and no more than a small percentage of any fraction chromatographed in a position suggesting reaggregation.

The collagen to gelatin transition of collagen extracted from the bones of lathyritic animals with 0.5 M acetic acid was studied in order to determine some of the physical properties of collagen from a tissue that normally calcifies. Melting curves for chick bone collagen in solution, as determined by the change in optical rotation with increasing temperature, are shown in Fig. 7. The midpoint between maximal and minimal specific optical rotation (θ_20_°_w) was 39.5° when the collagen was dissolved in 0.6 M acetic acid, and 41° when 0.15 M potassium acetate, pH 4.8, was the solvent. Identical values for the denaturation temperature of soluble chick skin collagen have been observed. The high initial specific rotation of the collagen in both solvents, as well as the sharp transition from collagen to gelatin, indicates that the material had been extracted in its native state.

The denaturation temperature of chick bone collagen is about 3° higher than that of mammalian collagens as represented by rat skin collagen (27). The specific rotations of both the native and the denatured forms are also higher. These differences are consistent with the higher total content of imino acid in chick bone collagen and are expected in view of the known correlations among body temperature, collagen stability, and imino acid content (16). The normal body temperature of the chick is about 41°, compared to about 37° for the white rat. The change in specific rotation on denaturation appears to be slightly greater for chick bone collagen than for rat skin collagen but is probably within experimental error, suggesting a similar content of helix. These data are summarized in Table V. Samples of rat skin and chick bone collagen were hydrolyzed at the same time and

-----

analyzed in successive runs to obtain the values for proline and hydroxyproline content. Otherwise the rather small differences would be within experimental error.

The amino acid composition of the collagen in successive extracts of lathyritic bones was identical except that the content of hydroxylysine increased in succeeding extracts and was highest in the insoluble residue. The increased hydroxylysine content was accompanied by an equivalent loss in lysine content, as summarized in Table VI. Since the more readily extractable collagen is generally of more recent origin than the insoluble collagen, these results suggested that lysine and hydroxylysine levels in bone collagen might vary normally with age. To investigate this possibility, diaphyseal areas from groups of 24 normal chicks ranging in age from 1 to 70 days were decalcified in EDTA solutions. The decalcified residues were then analyzed for amino acid composition after hydrolysis in 6 M HCl. As shown in Fig. 8, the hydroxylysine content underwent a progressive decrease from 11 to 6 residues per 1000 total residues between the 1st and the 49th day after hatching. Concomitantly, lysine content increased from 23 to 23 residues per 1000 total residues. Little further change could be observed to Day 70.

**DISCUSSION**

The experiments described here are interpreted by us as indicating that normal chick bone collagen is highly cross-linked. Only about 4% of the total collagen could be identified as α chains. This figure assumes that the collagen that was not extracted by 5 M guanidine-HCl contained little or no single-chain component. However, even if this fraction contained as much α chain as the extractable fraction, which is highly unlikely, the total content of α chain would be only about 20%. Furthermore, the data show that the observed α components are heterogeneous and may have arisen by degradation of larger
80 to 85% of the total collagen consisted of more than 75% \( \alpha \) chain; indicating a very low degree of cross-linking. It is important to reconcile these data, since the interpretation of Glimcher and Katz and of Glimcher et al. (18, 19) is that bone collagen is stabilized in the fibril largely by noncovalent forces, unlike most soft tissue collagens, in which covalent cross-links appear to be an important factor in intermolecular stabilization.

Probably the major difference can be ascribed to the different procedures used to prepare the samples. Glimcher and Katz (18) used stronger denaturing agents and longer periods of extraction than we used, and they were able to extract 80 to 85% of the collagen. It seems likely that extensive degradation may have occurred under these conditions. In support of this possibility are (a) the experiments described here showing that collagen kept in 5 M guanidine-HCl for 1 week becomes markedly heterogeneous and (b) the presence of material in the effluent of the agarose column having molecular weights less than \( \alpha \) chains. Furthermore, other experiments have shown the liability of \( \beta \) components (5, 28) and \( \alpha \) chains (8) when kept under denaturing conditions. It appears that the polypeptide chains, when not stabilized by secondary or tertiary structure, cleave at weak points under the stresses produced by thermal and mechanical agitation. We suggest that some of the products of degradation of cross-linked components have molecular weights and properties sufficiently close to those of unaltered \( \alpha \) chains to be confused with them.

A second important difference may be the method of quantitative determination. Glimcher and Katz (18) examined their samples by sedimentation velocity in the ultracentrifuge. This method does not readily lend itself to precise quantitative determination with the kind of samples studied; the method is also relatively insensitive to heterogeneity, owing to the large concentration dependence of sedimentation. The content of \( \alpha \) chain may therefore have been overestimated and its very marked heterogeneity may not have been apparent.

Our observations on lathyrisn also offer strong evidence that chick bone collagen normally is highly cross-linked. It has been shown that the collagen of animals given a lathyrogen such as \( \beta \)-aminopropionitrile becomes readily extractable (17) as a result of an inhibition of cross-linking (12, 29–31). Since it has been shown in the present study, as well as elsewhere (17, 20), that the bone collagen of lathyritic animals is readily extractable and has properties that are very similar to those of soft tissue collagen from lathyritic animals, it is reasonable to conclude that both hard and soft tissue collagens are stabilized by the same or similar cross-links. The only alternative is to attribute two alterations to the action of \( \beta \)-aminopropionitrile, as Glimcher et al. (20) have done. This seems unnecessarily complex in the light of present evidence.

Glimcher et al. (19) have reported that although bone collagen is not normally extractable by dilute acid, a large proportion can be solubilized as high molecular weight, but still native, aggregates after freezing and thawing. They interpreted this result in terms of strong noncovalent forces and their rupture by the mechanical introduction of water and acid between molecules. This explanation seemed to be required by their conclusion that bone collagen has relatively few covalent cross-links. However, if one accepts the suggestion offered here that bone collagen is highly cross-linked, it is at least as plausible that freezing and thawing can mechanically rupture a sufficient number of covalent bonds to solubilize or disperse a part of the collagen in the form of high molecular weight aggregates.

We do not mean to imply here that covalent cross-links are the only, or even the major, force stabilizing fibrous collagen. We do not have sufficient data or understanding to determine the relative importance of the many factors involved. However, we believe that the evidence strongly favors the conclusion, not only that hard and soft tissue collagens do not differ qualitatively at the level of structure so far examined, but also that quantitative differences are in the direction of a greater degree of covalent cross-linking in bone collagen than in most soft issue collagens.

Chick bone collagen has some unusual properties, as Glimcher and Katz (18), Glimcher et al. (19, 20), and Francois and Glimcher (21) have shown. For example, it does not swell in dilute acid but it can be extracted by denaturing agents. This is compatible with a high degree of covalent cross-linking but an unstable primary structure. Also, the guanidine-extracted fraction of chick bone collagen has a low content of \( \beta \) components, while mammalian skin collagen obtained in the same manner has a high content of \( \beta \) components (6, 15). This again suggests instability of denatured bone collagen, and suggests further that intermolecular cross-linking predominates in chick bone collagen while mammalian skin collagens are relatively richer in intramolecular cross-links. This property of chick bone collagen is shared by chick skin collagen,3 and is therefore a characteristic of the species rather than of the tissue.

The chain structure of chick bone collagen has been examined by Francois and Glimcher (21), with the use of molecular sieve chromatography, free flow electrophoresis, and CM-cellulose chromatography. They concluded that this collagen contains three different \( \alpha \) chains analogous to the \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) chains of codfish skin collagen (7, 8). Their criteria were first, a difference in electrophoretic and chromatographic behavior and second, an apparent difference in amino acid composition. Inasmuch as heterogeneity of the \( \alpha \) chains can be present normally in at least two ways (31, 32) or can arise artifactually as shown here (by guanidine treatment and perhaps in other ways), reliance must be placed on the amino acid analysis.2 In our opinion the apparent differences in composition between "\( \alpha_1 \)" and "\( \alpha_3 \)" reported by Francois and Glimcher (21) are within the normally expected experimental error. They are certainly much smaller than previously shown for the \( \alpha_1 \) and \( \alpha_3 \) chains of codfish skin collagen (7, 8). Furthermore, the \( \alpha_1 \) fraction of acid-extracted chick bone collagen from lathyritic animals as studied here shows no evidence of heterogeneity. We believe there is not sufficient evidence to conclude that the \( \alpha_1 \) fraction of chick bone collagen contains two fundamentally different species. The same arguments apply to calf skin collagen, studied by Francois and Glimcher (34) and by Heidrich and Wynston (35).

An additional finding of the present study is that the lysine to hydroxylysine ratio of chick bone collagen varies with age while...
the total content of the two amino acids is constant. This ratio is known to vary in collagens from a number of vertebrate species, as well as in collagens extracted from different tissues of the same species (37). Moreover, a progressive loss of hydroxylysine and an equivalent increase in lysine content accompanying advancing mineralization of the collagen of turkey tendon have previously been observed (38). In the latter study, no alteration in the content of lysine and hydroxylysine in tendon collagen of noncalcified areas was observed. The significance of these observations may be found in the carbohydrate which is bound to collagen through the side chain of hydroxylysine, probably in O-glycosidic linkage (39).

It is conceivable that during early development a different collagen is produced, which gradually replaces the collagen of the less mature tissue. If the collagen synthesized after hatching contained a greater proportion of its lysyl residues in sequences unavailable to the hydroxylating enzyme, then less lysine would be hydroxylated. However, if this were the case, it would be expected that the different sequences would cause a recognizable difference in composition. A more likely suggestion is that the collagen synthesized after hatching is the same as that elaborated in the embryonic bone and that the increasing ratio of lysine to hydroxylysine results from an alteration in the ability of the lysine hydroxylase to act on potentially available lysyl residues. The latter explanation is supported by the sequence studies of Bornstein (32) on a peptide derived by cyanogen bromide cleavage of α chains from rat skin and tail tendon collagens. In this work evidence was obtained that although a prolyl residue may be present in a sequence suitable for hydroxylation, other factors determine the extent of hydroxylation at a given site. Hydroxylysine, like hydroxyproline, is formed in peptide linkage by the action of lysine hydroxylase to act on potentially available lysyl residues. It is conceivable that during early development a different enzyme (40).

In the present studies on bone collagen, amino acid analyses of the total collagen showed no difference in over-all prolyl hydroxylation as a function of age. These differences would certainly have been detected had they occurred in the same proportion as that observed for lysyl residues, but would not have been detected if the change involved only a few residues per α chain. The amino acid compositions of α1 and α2 chains differ in a manner similar to that of the corresponding chains from other vertebrate collagens (4). The major differences are larger amounts of valine, isoleucine, leucine, and histidine and smaller amounts of glutamic acid and alanine in the α2 chain.

The denaturation curves of acid-soluble lathyritic chick bone collagen obtained in these studies clearly show that 20% of the total bone collagen can be extracted in the unimatured state. Since bones from lathyritic animals were as heavily calcified as bones from normal animals in cold 0.5 M acetic acid cannot be attributed to prior denaturation of the protein resulting from mineralization.

REFERENCES
Characterization of Chick Bone Collagen and Compositional Changes Associated with Maturation
E. J. Miller, G. R. Martin, K. A. Piez and M. J. Powers


Access the most updated version of this article at http://www.jbc.org/content/242/23/5481

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/242/23/5481.full.html#ref-list-1