**Human Skin Collagen**

**RELEASE BY PEPsin DIGESTION AND PREPONDERANCE IN FETAL LIFE**

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**SUMMARY**

Human dermis was digested with pepsin under conditions that maintain the helical conformation of the bulk of the collagen. Molecules containing \( \alpha(\text{III}) \) chains were separated from \( \alpha(\text{I}) \)-\( \alpha(\text{II}) \) collagen by differential salt precipitation at pH 7.5, with the use of the peptides produced by CNBr cleavage to monitor the composition of each fraction.

The isolated molecules are composed of three \( \alpha(\text{III}) \) chains, and these chains are cross-linked by disulfide bonds. Exposure to dithiothreitol liberates the three apparently identical chains, and these elute on carboxymethylcellulose chromatography at a position intermediate between \( \alpha(\text{I}) \) and \( \alpha(\text{II}) \). The pepsin-resistant portion of these chains is the same size as that of previously isolated collagen chains.

Human dermis was also digested with CNBr, and resultant \( \alpha(\text{I}) \) and \( \alpha(\text{III}) \) peptides were separated chromatographically. The relative quantities of these peptides indicate that \( \alpha(\text{III}) \) chains predominate in early fetal skin, but by birth and in later life \( \alpha(\text{I}) \) chains are approximately 3 times as plentiful.

Collagen extracted from human skin has been well characterized in its chain composition (2) and also at the level of CNBr peptides (3, 4). This molecule is composed of two identical \( \alpha(\text{I}) \) chains and a differing, \( \alpha(\text{II}) \) chain and is closely homologous to collagen extracted from skin, bone, and tendon of several species including baboon (4), chick (5-8), rat (9-11), guinea pig (12), calf (13-15), and rabbit (16, 17).

The collagen extracted from cartilage, by contrast, is composed of three identical \( \alpha(\text{I}) \) chains and is closely homologous to collagen extracted from skin, bone, and tendon of several species including baboon (4), chick (5-8), rat (9-11), guinea pig (12), calf (13-15), and rabbit (16, 17).

Human dermis was obtained, \( \alpha(\text{III}) \) chains being predominant in fetal skin. The chains are the same length as the \( \alpha(\text{I}) \) and \( \alpha(\text{II}) \) chains isolated from the same digests but differ markedly in their elution in carboxymethylcellulose chromatography and in their CNBr peptides. Unlike collagen molecules previously isolated from the skin of vertebrates, these molecules contain chains that are cross-linked by disulfide bonds. In addition, the relative quantities of peptides derived from CNBr digestion of skin have been studied. The ratio of the two types of \( \alpha(\text{I}) \) chains varies with the age of the individual from whom the dermis was obtained, \( \alpha(\text{III}) \) chains being predominant in fetal life and \( \alpha(\text{I}) \) chains being the more plentiful in adult life.

**MATERIALS AND METHODS**

**Skin Samples**—Human skin used for pepsin digestion was obtained from the trunk and proximal extremities of infants at the time of autopsy. Abdominal skin removed at the time of routine autopsy was used for most determinations of chain ratios at different ages. In one adult, skin samples from both the abdomen and the leg were obtained; in one 5-year-old girl, skin was obtained from a normal appearing area of the calf of a leg amputated for treatment of a soft tissue sarcoma of the thigh, and in small fetuses the skin of the whole trunk and proximal extremities were utilized. The fetuses were obtained at hysterotomy performed for therapeutic abortion; most of the newborn babies died of pulmonary failure, and the adults died of a variety of causes, most commonly the complications of malignant disease or atherosclerosis.

The subcutaneous fat was scraped off the dermis, and the skin was cut into pieces 5 to 10 mm. Skin was either lyophilized directly or was extracted at 4°C with two portions of 1 m NaCl-0.05 M Tris at pH 7.5 for 1 week and then with five changes of 0.5 M acetic acid over 4 weeks. The remaining insoluble residue was lyophilized.

**Cleavage with Pepsin and Isolation of Molecular Species**—One hundred milligrams of pepsin (Worthington, twice recrystallized, 3000 units per mg) were dissolved in 200 ml of 0.4 M acetic acid at 4°C. To this was added 1 g of the finely diced insoluble dermis.
and the suspension was stirred at 18°C for 22 hours. Subsequent manipulations were performed at 4°C. Undigested tissue was removed by centrifugation for 1 hour at 16,000 × g and lyophilized. The supernatant was filtered (Whatman No. 1 filter paper), and the collagen was precipitated twice by dialysis against 0.02 M NaHPO4 at 4°C, then once by dialysis against 0.5 M acetic acid containing 0.86 M NaCl. Each precipitate was dissolved in 0.1 M acetic acid.

The last solution was adjusted to pH 7.5 by dialysis against 0.05 M Tris, pH 7.5, containing 0.2 M NaCl. Fractions were then collected by dialysis against 0.05 M Tris, pH 7.5, containing progressively higher concentrations of NaCl: 0.86, 1.38, 1.71, 2.05, 2.39, and 2.56 M. The material precipitating at 1.71 M was reprecipitated at the same salt concentration. Each precipitate was collected by centrifugation for 1½ hours at 35,000 × g, dissolved in 0.5 M acetic acid, dialyzed against 0.5 M acetic acid, and lyophilized.

Cleavage with CNBr—Samples of unextracted skin or of insoluble dermis (50 to 500 mg) were suspended in 25 to 50 ml of 70% formic acid. The suspension or solution was flushed with nitrogen, and a weight of CNBr 2 to 3 times the volume were reduced for this smaller column to 50 ml per hour and was lyophilized separately. The liberated peptides were reprecipitated from formic acid and CNBr by passage over a column of Bio-Gel P-2 (50 to 100 mesh, Bio-Rad) and were lyophilized.

CM-Cellulose Chromatography—Collagen molecules were denatured and chromatographed on a column (9 × 90 mm) of CM-cellulose (Whatman No. CM-52, microgranular, capacity 1.0 meq per g) at 43°C using a NaCl gradient in sodium acetate as described previously (4), except that the flow rate and gradient volume were reduced for this smaller column to 50 ml per hour and 450 ml, respectively. In some instances, dithiothreitol (Calbiochem) was added to a concentration of 0.001 M to the deacreated buffers.

Peptides liberated by CNBr digestion were chromatographed on the same column (25- to 50-mg samples) or on a column (25 × 90 mm) (100- to 200-mg samples) at 43°C. Samples were dissolved in 4 to 10 ml of starting buffer (0.02 M sodium citrate, 0.02 M NaCl, adjusted to pH 3.8 with citric acid). The solutions were clarified by centrifugation at 35,000 × g for 40 min and passage through a medium porosity sintered glass filter and were then applied to the column equilibrated with the same buffer. Peptides were eluted from the column with a linear gradient prepared from 250 ml of starting buffer and 250 ml of limit buffer (0.02 M sodium citrate, 0.20 M NaCl adjusted to pH 3.8 with citric acid) at a flow rate of 50 ml per hour and from the larger column with a gradient volume and flow rate 4 times as large.

The effluent from all columns was monitored continuously at 220 to 230 nm by means of a Beckman DB-GT spectrophotometer. Appropriate fractions were lyophilized, separated from salts by passage over columns of Bio-Gel P-2, and were relyophilized.

Agarose Chromatography—Separation and molecular weight determinations of denatured collagen chains were achieved on calibrated columns (1.8 × 230 cm) of agarose beads (Bio-Gel A-5m or A-15m, each 200 to 400 mesh, from Bio-Rad) at 25°C (25). Samples were applied to the column at a denaturation to liquid nitrogen concentration. Dithiothreitol, 0.001 M, was added to the eluting solution of 1 M CaCl2, 0.05 M Tris at pH 7.5 for some chromatograms. Blue dextran 2000 (Pharmacia) was used to determine the excluded volumes of these columns.

Separation, molecular weight determination, and determination of relative amounts of CNBr-labeled peptides derived from CM-cellulose chromatography were achieved on a column (1.8 × 230 cm) of agarose beads (Bio-Gel A-1.5, 200 to 400 mesh) at 23°C.

Calculation of Peptide Ratios—Comparison of the relative quantities of peptides present was performed by tracing the appropriate portions of chromatograms onto paper, cutting out the traced outlines, and weighing the individual pieces. These weights were divided by the molecular weights of the peptides to give estimates of the relative molar ratios of the molecules of each species that were present. In the case of α(1)-CB-3, the amount of peptide migrating as the uncleaved peptide α(1)-CB(3) was added to that migrating as the fully cleaved peptide.

Amino Acid Analysis—Samples were hydrolyzed as described previously (9). Amino acid analyses were carried out on a Beckman model 117 amino acid analyzer with a 4-buffer elution system similar to that described previously (24). These were kindly performed by Mr. Guy Hawkins in the laboratory of Dr. George Martin, National Institute of Dental Research, Bethesda, Maryland.

RESULTS

Pepsin Digests and Differential Salt Precipitation of Components—After pepsin treatment, 10 to 14% of the original weight of skin digested was recovered in the 16,000 × g pellet. The peptides liberated from this pepsin-insoluble material by CNBr cleavage were chromatographed on CM-cellulose, but only very poor resolution of peptides could be obtained. This precluded the determination of the chain ratios of the material not solubilized by pepsin treatment.

The material twice precipitated by dialysis against 0.02 M NaHPO4, comprised approximately 20% of the original material digested. No attempt was made to recover the remaining material from the large volumes of dialysis fluid. (In experiments in which only pepsin was added without skin to the original incubation mixture, no precipitate was obtained upon dialysis against 0.02 M NaHPO4.) During subsequent fractionation steps, the solutions were cloudy until dialyzed against 1.37 M NaCl-0.05 M Tris at pH 7.5. At this salt concentration, the supernatant was clear and colorless.

Subsequent dialysis against 1.71 M NaCl precipitated most of the α(1)(III), while most of the α(1) and α(2) remained in solution. After reprecipitation under the same conditions, α(1)(III) comprised at least 85% of the material. This was determined by digesting an aliquot with CNBr and separating the resultant peptides (Fig. 1, top, Fig. 2, top). This fraction contained approximately 2% of the original skin that was digested with pepsin. Collagen precipitating at 2.56 M NaCl contained only α(1)(I)α(2) (Fig. 1, bottom, Fig. 2, bottom).

The CM-cellulose chromatographic pattern of CNBr-released peptides of α(1)(I) (Fig. 1, top) clearly differed from that of α(1)(I)α(2) (Fig. 1, bottom). The presence of α(1)(I)-CB3 (80 ml) and α(1)(I)-CB(4,5) (165 ml) as well as the absence of α(1)-CB3 (105 ml) was evident. The peak eluting at 220 to 240 ml contained a previously undescribed peptide of 113 amino acids designated here, for convenience, as α(1)(III)-CB13 (Fig. 1, top, Fig. 2, top). It contained hydroxyproline, and one-third of its residues were glycine (Table I). It had no obvious homology with CNBr peptides of human α(1)I or α2 chains (3, 4), chick α(1)(II) chains (19), or the α chain of bovine lens capsule (26). The final large absorbance peak (275 to 350 ml, Fig. 1, top) contained material that elutes from agarose A-1.5m at a site corresponding to a molecular weight of 80,000, a pattern that differed from agarose chromatography of the same region from α(1)(I)α(2) CNBr peptide chromatograms (Fig. 1, bottom), in which peptides of molecular weights of 60,000 (α2-CB-3(5)-1) and 30,000 (α2-CB-5) were separated (4).

Agarose Chromatography of Denatured Molecules—Material precipitated by dialysis against 0.02 M NaHPO4 was chromatographed on agarose A-1.5m at a site corresponding to a molecule with a molecular weight of 80,000, a pattern that differed from agarose chromatography of the same region from α(1)(I)α(2) CNBr peptide chromatograms (Fig. 1, bottom), in which peptides of molecular weights of 60,000 (α2-CB-3(5)-1) and 30,000 (α2-CB-5) were separated (4).

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...by NaCl precipitation and containing approximately 85% \( \alpha(III) \) collagen yielded trimers and larger species with only very small amounts of \( \alpha \)-sized material (Fig. 3, bottom). Another aliquot of the 0.02 M \( \text{NaHPO}_4 \) precipitate was chromatographed on agarose A-5m with eluent to which 0.001 M dithiothreitol had been added. The trimer and larger molecular weight material decreased markedly in amount, and more material of \( \alpha \) size appeared (not shown in Fig. 3).

**CM-Cellulose Chromatography of Denatured Molecules**—
Material precipitating on dialysis against 0.02 M \( \text{NaHPO}_4 \) was chromatographed on CM-cellulose. Although heterogeneity of the \( \alpha \) and \( \alpha_{11} \) regions was evident, no \( \alpha(III) \) peptides were detected after CNBr cleavage of that material. All the \( \alpha(III) \) eluted in the position expected for \( \beta_{11} \), and \( \alpha(III) \) purified by salt precipitation eluted at the same site (Fig. 4, top). When the latter material was then chromatographed on agarose A-5m, approximately one-half eluted at the excluded volume and one-half at a site expected for a trimer (Fig. 5). No material eluted at a site corresponding to a molecular weight smaller than 280,000. Each of these two fractions from agarose A-5m was rechromatographed on the same column and eluted with the same solution, to which 0.001 M dithiothreitol had been added; in each instance most of the material then eluted at a site expected for \( \alpha \) chains (Fig. 6). Amino acid analyses of the constituent in the two peaks from the initial agarose chromatogram and of material from the second peak that migrated as \( \alpha \) chains on rechromatography were similar; each contained one-third glycine, high amounts of hydroxyproline, and significant amounts of cysteine (Table I).

A separate sample of the \( \alpha(III) \) purified by salt precipitation was chromatographed on CM-cellulose, and the material producing the single large absorbance peak (Fig. 4, top) was rechromatographed on the same CM-cellulose column with the use of a gradient that differed only by the addition of 0.001 M dithiothreitol. Again, a single peak of absorbance at the same site was seen. No material appeared in the \( \alpha(1) \) or \( \alpha_{2} \) regions. However, when that material was lyophilized, separated from salts by passage over Bio-Gel P-2, and chromatographed on agarose A-5m without dithiothreitol, the reduction in size of most of the material from trimer to \( \alpha \) size was evident. As a control, acid-soluble human skin collagen containing only \( \alpha(1) \), \( \alpha_{2} \) molecules was chromatographed on CM-cellulose,

![Fig. 1. CM-cellulose chromatograms of the CNBr peptides from collagen released from dermis by pepsin digestion and precipitated twice by dialysis against 1.71 M NaCl-0.05 M Tris, pH 7.5, top, and collagen released from insoluble dermis by pepsin digestion and precipitated once by dialysis against 2.56 M NaCl-0.05 M Tris, pH 7.5, bottom.](http://www.jbc.org/)

**Fig. 2.** Agarose A-1.5m chromatograms of the CNBr peptides eluting between 215 and 246 ml on CM-cellulose as shown in Fig. 1. Top and bottom are as in Fig. 1. The material eluting at 240 ml is \( \alpha(1)-\text{CB(83)} \), at 270 ml is \( \alpha(1)-\text{CB8} \), and at 330 ml is \( \alpha(III)-\text{CB113} \).

**Table I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxyproline</td>
<td>14</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6 (5.9)</td>
</tr>
<tr>
<td>Threonine</td>
<td>15</td>
</tr>
<tr>
<td>Serine</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9 (8.9)</td>
</tr>
<tr>
<td>Proline</td>
<td>102</td>
</tr>
<tr>
<td>Glycine</td>
<td>355</td>
</tr>
<tr>
<td>Alanine</td>
<td>92</td>
</tr>
<tr>
<td>1/2 Cys</td>
<td>2 (2.4)</td>
</tr>
<tr>
<td>Valine</td>
<td>16</td>
</tr>
<tr>
<td>Methionine</td>
<td>7 (6.8)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>21</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>8 (7.6)</td>
</tr>
<tr>
<td>Lysine</td>
<td>30</td>
</tr>
<tr>
<td>Histidine</td>
<td>6 (6.1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>40</td>
</tr>
<tr>
<td>Homoserine</td>
<td>1 (0.7)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Residues per peptide \( \alpha(III)-\text{CB113} \) or residues per 1000 \( \alpha(III) \), rounded off to the nearest whole number. Actual values are listed in those cases in which fewer than 10 residues were found. Less than 0.2 residues were found of those amino acids not listed.

<sup>b</sup> These values represent the averages of five separate determinations, each of a different preparation. No significant differences were found for preparations in which the component chains had been separated by exposure to dithiothreitol as compared with those in which the disulfide bonds were intact.

<sup>c</sup> Determination of half-cystine as cysteic acid after performic acid oxidation (25) gave a value of 1.9 residues per 1000.
and the material migrating as $\beta_{12}$ was rechromatographed on the same column with the use of a gradient with added di-thiothreitol. The elution position was unchanged, and agarose chromatography of that material revealed only dimer-sized components.

**CNBr Digestion of Skin**—Recoveries of solubilized peptides released by CNBr digestion of skin averaged 80% of the dried weight of the sample digested. Recoveries from digests of unextracted samples and from samples of insoluble dermis did not differ significantly, averaging 81% and 79%, respectively. Also, recoveries did not differ significantly among the samples obtained from the various age groups studied: 76% from the fetal skin, 81% from the newborn skin, and 80% from the adult skin. By comparison, CNBr digestion of soluble human skin collagen generally yields a weight of digested peptides 90% of the initial substrate weight. Amino acid analysis of material insoluble after CNBr digestion yielded values for 4-hydroxyproline of 45 to 76 residues per 1000 residues and for glycine of 122 to 207 residues per 1000 residues, suggesting that approximately one-half of the digestion-resistant material was collagen. This material is also enriched, compared with whole dermis, in the elastin cross-links desmosine and isodesmosine, a finding consistent with the lack of methionyl residues in elastin (27).

**Separation and Detection of CNBr Peptides of $\alpha(1)$ and $\alpha(3)$—**CM-cellulose chromatograms of the CNBr-derived peptides of insoluble dermis partially resolved the mixture of $\alpha(1)$, $\alpha2$, and $\alpha(3)$ peptides. As described previously (22), the presence of $\alpha(3)$-CB3 and $\alpha(3)$-CB(4,5) was apparent on such chromatograms (Fig. 7, middle, Regions A and B, respectively). Agarose chromatography of the peptides eluting in Region C (Fig. 7, middle) separated well $\alpha(1)$-CB(8-3) and $\alpha(1)$-CB8 from $\alpha(3)$-CB13 (Fig. 8).

**Chain Ratios in Various Skin Samples—**CM-cellulose chromatograms of CNBr peptides from fetal, newborn, and adult skin demonstrated differences in the ratios of $\alpha(1)$ to $\alpha(3)$ peptides with a relative abundance of $\alpha(3)$-CB3 and $\alpha(3)$-CB(4,5) in the younger specimens and a relative paucity of these peptides in the adult tissue (Fig. 7). This can be seen in Fig. 7 most directly by comparing the size of Peak A with the size of the peak that follows it. The latter contains the homologous peptide $\alpha(1)$-CB3. The $\alpha(3)$-CB3 peak (A) is...

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**Fig. 3.** Agarose A-15m chromatogram of denatured collagen released from insoluble dermis by pepsin digestion. Top, precipitated twice by dialysis against 0.02 M NaHPO$_4$. Bottom, precipitated twice by dialysis against 1.71 M NaCl-0.05 M Tris, pH 7.5.

**Fig. 4.** CM-cellulose chromatogram of denatured collagen. Top, released from insoluble dermis by pepsin digestion and precipitated twice by dialysis against 1.71 M NaCl-0.05 M Tris, pH 7.5. Middle, released from insoluble dermis by pepsin digestion and precipitated by dialysis against 2.56 M NaCl-0.05 M Tris, pH 7.5. Bottom, 0.5 M acetic acid-extracted human skin collagen. $S =$ sample applied; $G =$ gradient started.

**Fig. 5.** Agarose A-5m chromatogram of collagen released from insoluble dermis by pepsin and purified by differential salt precipitation at 1.71 M NaCl-0.05 M Tris, pH 7.5, and subsequent CM-cellulose chromatography (Fig. 4, top). Excluded, $\beta$, and $\alpha$ mark the expected elution volumes on this column for blue dextran 2000, $\beta$ components, and $\alpha$ or $\alpha2$ chains of 0.5 M acetic acid-extracted human skin collagen.

**Fig. 6.** Agarose A-5m chromatogram of material eluting at 175 to 190 ml as shown in Fig. 5. Conditions were the same as in Fig. 5 except that 0.001 M dithiothreitol was added to the eluting fluid.
TABLE II
Ratios of $\alpha_1(I)$ to $\alpha_1(III)$

<table>
<thead>
<tr>
<th>Age of patient</th>
<th>Number of patients</th>
<th>Unextracted skin</th>
<th>Insoluble dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-Week fetus</td>
<td>2</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>18-Week fetus</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>22-Week fetus</td>
<td>1</td>
<td>1.6</td>
<td>0.8, 1.1</td>
</tr>
<tr>
<td>Newborn infant</td>
<td>2</td>
<td>2.7</td>
<td>1.4, 1.5, 1.6, 1.6,</td>
</tr>
<tr>
<td>3 Months</td>
<td>1</td>
<td>2.5, 2.6, 2.7</td>
<td>1.6, 1.7, 1.7, 1.8, 1.8, 1.8, 1.8, 1.9, 1.9</td>
</tr>
<tr>
<td>8 Years</td>
<td>1</td>
<td>2.6, 3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>20 Years</td>
<td>1</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>30 Years</td>
<td>1</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>40 Years</td>
<td>2</td>
<td>2.7, 3.0</td>
<td></td>
</tr>
<tr>
<td>43 Years</td>
<td>1</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>56 Years</td>
<td>1</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>60 Years</td>
<td>1</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>63 Years</td>
<td>1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>75 Years</td>
<td>2</td>
<td>2.7, 4.1, 2.7, 3.1</td>
<td></td>
</tr>
</tbody>
</table>

* Fetal ages were approximate and were estimated both from the time elapsed from the mother's last menstrual flow, when this was known, and from the length and weight of the fetus. The amount of skin obtained from a single 12-week-old fetus studied was insufficient to permit an assessment of chain ratio.

ratios were calculated from agarose chromatograms of peptides eluting in region C from CM-cellulose chromatography.

The molar ratios of $\alpha_1(I)$ to $\alpha_1(III)$ calculated from the duplicate agarose chromatograms were 1.4:1, 1.6:1, 1.7:1, and 1.8:1 from one digest, and the ratios from the separate CNBr digestion, CM-cellulose chromatogram, and duplicate agarose chromatograms were 1.7:1, 1.7:1, and 1.8:1.

2. Peptides derived from agarose chromatography of Region C were collected, desalted, and weighed. Using the measured weights, a molar ratio of $\alpha_1(I)$ to $\alpha_1(III)$ of 1.8:1 was calculated. By comparison, a molar ratio of 2.0:1 was calculated from the weights of the appropriately traced peaks of absorbance, thus suggesting that the absorbance was a reliable estimate of the total amount of each peptide present.

3. Duplicate CM-cellulose chromatography was performed on aliquots from a single CNBr digest. In one instance, Region C was limited to 10 9.5-ml fractions (95 ml); in the other instance, two 9.5-ml fractions on either side of Region C were added to Region C (total volume 133 ml). The molar ratios of $\alpha_1(I)$ to $\alpha_1(III)$ calculated from agarose chromatograms of these samples were 1.7:1 and 1.9:1, respectively, thus suggesting that variability in selecting the limits of Region C did not cause marked changes in the molar ratios calculated.

4. One skin sample was divided into two portions. One aliquot was lyophilized directly; the other aliquot was stirred in 0.5 M acetic acid for 1 month, following which the residue and the acid-extracting solution were lyophilized together. Each aliquot was subjected to CNBr digestion, CM-cellulose chromatography, and agarose chromatography of Region C. The calculated molar ratios agreed closely, being 2.5:1 for the directly lyophilized sample and 2.2:1 for the acid-treated skin, thus suggesting that prolonged exposure to dilute acetic acid alone did not of itself change the observed chain ratios.
5. Insoluble dermis from the abdomen and thigh of a 40-year-old woman gave calculated molar ratios of 3.9:1 and 3.8:1, respectively. Unextracted skin from the back and chest of one newborn infant gave molar ratios of 2.2:1 and 1.8:1, respectively. These results suggest that the ratios of chains do not vary markedly between at least these different sites. Also, no difference was observed in chain ratios in dermis from males or females of the same age.

**DISCUSSION**

*Isolation of $[\alpha_1(III)]_3$*- This paper details the isolation from pepsin digests of human dermis of molecules containing $\alpha_1(III)$ chains. Although experiments reported here were performed with material released by digestion at $18^\circ$, similar results were obtained when the digestion was performed at $4^\circ$ for the same length of time.

It has been evident for some time that the bulk of the collagen molecule is resistant to pepsin digestion so long as the triple helix is intact. Pepsin does cleave nonhelical terminal segments from the molecule (28). The liberation of most of the collagen from otherwise insoluble tissue by pepsin digestion is compatible with current concepts that much of the covalent cross-linking between collagen molecules occurs at nonhelical sites (29-33). Similar liberation of collagen from basement membrane (34) and cartilage (24) has been effected by pepsin digestion of otherwise insoluble preparations.

The preservation of the helix in the material released by pepsin digestion of skin is indicated by the resistance to the initial pepsin digestion and by precipitation on dialysis against 0.02 M Na$_2$HPO$_4$ (35). Also indicative are the ability to form segment long spacing (SLS) crystalloids, and the typically high optical rotation with loss upon heating to a temperature ($30^\circ$) that is the same as for $[\alpha_1(III)]_2$ isolated from pepsin digests.

The finding of significant amounts of cysteine was unexpected. This amino acid has been found in vertebrate collagens only in basement membrane (34) and in the nonhelical NH$_2$-terminal extensions of procollagen (36-40). It is unlikely that the $\alpha_1(III)$ could be derived from basement membrane of epidermis or epidermal appendages or of dermal blood vessels, since it comprises approximately one-quarter to one-half of the total skin collagen. This quantity is more than could be ascribed to the amount of basement membrane recognizable histologically. Also, the basement membrane preparations studied have been very rich in hydroxylysine, whereas $\alpha_1(III)$ contains only as much of this residue as is found in $[\alpha_1(II)]_2$ (3, 4). Lastly, the peptides derived by CNBr digestion of basement membrane do not resemble those derived from $\alpha_1(III)$ (26).

It is unlikely that the cysteine is present in a nonhelical procollagen peptide, since the latter are removed by pepsin (37, 41). The presence of some $\beta$ components of $[\alpha_1(II)]_2$ isolated from the same pepsin digests, however, suggests the possibility that some nonhelical portion may survive this digestion. Since the intramolecular bond linking two chains in soluble collagen is located in the nonhelical NH$_2$-terminal region. Alternatively, the persistence of $\beta$ components may be due to intermolecular cross-linking at a helical site. Since the molecular weight of the $\alpha_1(III)$ collagen falls from approximately 280,000 to approximately 93,000 on addition of 0.001 M dithiothreitol to the eluting buffer on agarose chromatography, some of the cysteine residues must form disulfide cross-links. It is tempting to speculate that the inability to extract $\alpha_1(III)$ from the skin even of lathyritic animals may be due to the persistence of disulfide bonds that are intermolecular. This possibility is supported by the liberation of $\alpha$-sized moieties on exposure to dithiothreitol of the material larger than trimers (Fig. 5, excluded material).

Cysteine is also present in the collagen of *Ascaris lumbricoides* cuticle. The chains of that collagen form an intrachain helix by folding back on themselves and are linked together by disulfide bonds (42, 43). The chains are, however, smaller than the $\alpha_1(III)$ chains isolated from human skin, having a molecular weight of 62,000.

The chain composition of this collagen is probably $[\alpha_1(III)]_3$. This is suggested by the failure to liberate chains migrating in the $\alpha_1(II)$-$\alpha_1(II)$ or $\alpha_2$ regions on CM-cellulose chromatography performed with dithiothreitol added to the gradient buffers. As judged by subsequent agarose chromatography, trimers are cleaved to $\alpha$ chains under these conditions. The CM-cellulose chromatogram of the CNBr-released peptides from the $\alpha_1(III)$-containing fraction does contain material that elutes in the position expected for $\alpha_2$-derived peptides. However, the molecular weight of 80,000, with absence of 60,000 and 30,000 molecular weight peptides, suggests that this material is not derived from the known $\alpha_2$ chain. Since only $\alpha_1(III)$ chains seem to be present, and since most of the $\alpha_1(III)$ is present in these preparations in a moiety of a molecular weight of 280,000 and can be dissociated by dithiothreitol to a molecular weight of approximately 93,000, the most likely chain composition for this molecule is $[\alpha_1(III)]_3$.

The separation of $[\alpha_1(I)]_2\alpha_2$ from $[\alpha_1(II)]_3$ by differential salt precipitation at pH 7.6 in phosphate buffer has been reported (44). The former precipitates at 2.2 M NaCl; the latter remains in solution and can be precipitated at higher NaCl concentration. Using Tris buffer at pH 7.5, it has been demonstrated here that pepsin-digested $[\alpha_1(III)]_3$ precipitates at the lower salt concentration of 1.7 M NaCl. This suggests that it might be possible to separate all three of these types of collagen by differential salt precipitation were such a separation necessary.

$\alpha_1(III)$ Preponderance in Fetal Skin—These studies demonstrated that $\alpha_1(III)$ chains are the most plentiful type of collagen in the skin of early fetuses, but comprise a much smaller fraction of skin collagen in children and adults. The reproducibility of the methods used seems quite adequate for this conclusion.

The relative paucity of $\alpha_1(III)$-CB113 in adult skin is not due solely to a dilutional effect with maintenance of the same total quantity of $\alpha_1(III)$ with an increasing amount of $\alpha_1(I)$, because the quantity present in a small fetus is many times less than the quantity of $\alpha_1(III)$ present in even a small part of the skin of a child or an adult. It is unknown whether this ratio is controlled by differing rates of synthesis, of degradation, or both. The possibility that different collagen molecules may be degraded independently is suggested by the demonstration that $[\alpha_1(I)]_2$ molecules of cartilage resist attack by collagenase preparations that are active against $[\alpha_1(I)]_2\alpha_2$ molecules (45).

Trelstad et al. found in neutral salt extracts of skin of lathyritic chick embryos a collagen species containing no $\alpha_2$ chains (46). They postulated on the basis of amino acid analyses that this was composed of chains differing from $\alpha_1(I)$ and $\alpha_1(II)$ and proposed the designation $\alpha_1(III)$. This species was not found in extracts from skin of lathyritic 3-week-old chicks. The exact identity of the molecule they described is not certain, but it is

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1 Ervin H. Epstein, Jr., and Kimie Fukuyama, unpublished observations.
2 Ervin H. Epstein, Jr., and Jen T. Yang, unpublished observations.
likely that it is not the same as the α1(III) described herein because there are significant differences in the amino acid composition of the two preparations.

The conclusion that skin collagen varies with age was also reached by study of the reducible aldimine cross-links of skin from fetuses and newborns (47). In fetal skin from humans, chickens, or calves, the major such cross-link is derived from 2 hydroxylysine residues, whereas in more mature skin the major such cross-link is derived from 1 hydroxylysine and 1 lysine residue. One explanation suggested for this difference in cross-links was a difference in primary structure of the collagen at different ages, but age-related differences in hydroxylation of the same lysine residue could not be excluded. No evidence is available, however, that the two cross-links are indeed derived from [α1(I)]ε2 and [α1(III)]ε2 molecules.

The realization that various tissues may contain genetically distinct collagen molecules is a relatively recent one. At this time, no clear data have been presented linking differences in collagen molecules to differences in normal tissue structure or function. The finding of a preponderance of α1(III) chains in early fetal skin and a relative paucity of these chains postnatally might suggest that their presence could be important in facilitating remodeling and growth of the skin. However, other tissues including tendon and deep muscle fascia contain only [α1(I)]ε2 collagen molecules in children, thus suggesting that α1(III) chains are not necessary for growth at least in these tissues.

If molecules containing α1(III) functioned in maintenance of the epidermis, one might also expect a preponderance of these molecules in the thinner skin of the fetus, where relatively more of the dermis lies close to the epidermis. That explanation would also account for its absence in tissues such as tendon that do not support an epithelium, as well as its presence in tissues with an epithelium or endothelium, including rat lung, human placental membranes, and human aorta. The same considerations would apply if [α1(III)]ε2 were produced by the epidermis, it now being clear that epithelia are capable of secreting collagen (48). Studies to localize the α1(III) chains within the dermis are now in progress.

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