Structure of Two Histidine-containing Cross-Links from Collagen*

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SUMMARY

Reduction of insoluble collagens from bone, skin, and tendon with NaB₃H₄ produced several radioactive substances which were isolated from acid hydrolysates of the protein. One of these substances, aldol-histidine, was most abundant in cow skin collagen and also appeared in reconstituted fibrils of purified cow skin tropocollagen. Another compound, histidino-hydroxymerodesmosine, was abundant in all of the collagens studied, and it appeared to be structurally related to aldol-histidine. The postulated structures of both compounds were obtained primarily by high and low resolution mass spectrometry and by high resolution nuclear magnetic resonance spectrometry. Ancillary data were provided by ultraviolet spectrometry and by colorimetric methods.

Both compounds were present in partially purified, large peptides obtained from cyanogen bromide digests of insoluble collagen and of reconstituted collagen fibrils. These results suggest that aldol-histidine and histidino-hydroxymerodesmosine serve as cross-links in collagen, potentially uniting three or four polypeptide chains, respectively.

EXPERIMENTAL PROCEDURES

Preparative Steps—Collagen was extracted from skin and tendon using cold 0.1 M acetic acid (three consecutive extracts of 24 hours each) and was purified as described (1). The residual tissue was termed “insoluble collagen” and was treated with NaB₃H₄ by a procedure similar to that used for reconstituted collagen fibrils (1). Specifically, the particulate insoluble wet collagen was suspended in 500 volumes (v/w) of sodium phosphate buffer, pH 7.5, ionic strength 0.16, at 37°C. A drop of Antifoam B (Dow Corning) was mixed in, followed by rapid addition of a 100-fold molar excess (relative to the moles of collagen) of NaB₃H₄ in 0.01 M NaOH just prior to use. Following incubation at 37°C for 30 min the protein frag-
ments were harvested by filtration. They were then exhaustively washed by repeated cycles of suspension in distilled water and filtration. The product was then lyophilized. The pulverized tibias from young calves (2 to 3 days old) were demineralized with 0.6 M Na2EDTA, pH 8.1, at 4°C by shaking for several weeks in frequently replenished solvent. The final product contained minimal amounts of residual mineral as determined by ashing; it was treated with NaBH₄, by the same method used for soft tissue collagen. Reduction of collagen with NaBD₄ was carried out in similar fashion (14). In all cases, the pH of the reaction mixture did not change.

The tritiated proteins were hydrolyzed in 3 N HCl by refluxing for 48 hours, and the reaction mixture was taken to dryness in a rotary evaporator. Up to 10 g of hydrolysate, adjusted to pH 2, were applied to a column (2.5 cm × 60 cm) of Dowex 50-X8, 200 to 400 mesh, equilibrated with 0.25 M potassium formate, pH 2.9, and maintained at 50°C. A linear gradient between the potassium formate buffer and 0.75 M potassium acetate was then used, over a total volume of 2.5 liters. The column effluent was monitored for radioactivity using a toluene-based scintillation fluid containing Beckman Biosolv-3. The peak areas were pooled and titrated with 60% HClO₄, at 4°C until precipitation of the KClO₄ ceased; the precipitate was removed by centrifugation at 4°C. The supernatant fluid was concentrated by rotary evaporation, applied to a Dowex 50-X8 column (II form) and the radioactive compounds were eluted with 2 M NH₄OH. This solution was dried by rotary evaporation at 40°C, then the residue was dissolved in 0.01 M HCl and applied to a column (0.9 cm × 21 cm) of Bio-Rad Aminex A-5, which was equilibrated with 0.2 M pyridine acetate, pH 3.5, and maintained at 30°C. The column was developed with a linear gradient between the equilibration buffer and 0.8 M pyridine acetate, pH 5.2, over a total volume of 200 ml. The fractions were monitored for radioactivity, and the pooled fractions were dried in a rotary evaporator. Additional purifications, when necessary, were carried out by using the A-5 resin column with other pyridine acetate gradients or by collecting from the amino acid analyzer columns, using the ordinary citrate buffers. The purity of the isolated compounds was evaluated by amino acid analysis employing a column (0.9 cm × 13 cm) of Beckman PA-35 resin, maintained at 50°C and developed with 0.35 M sodium citrate, pH 5.25. By pooling several fractions, as much as 1 to 2 mg of pure radioactive material could be isolated from 20 to 25 g of collagen. Over-all yields were not determined.

Cyanogen bromide digestion of either NaBH₄-reduced insoluble collagen or NaBD₄-reduced reconstituted fibrils (1) was carried out by suspending the lyophilized protein in 70% formic acid, adding an equal weight of CNDI and incubating at room temperature for 90 min. This time was selected because control studies showed that: (a) all of the protein dissolved, (b) all methionine residues were converted to homoserine residues, (c) the SDS-acrylamide gel pattern did not alter after 90 min of digestion. Subsequently, the digest was passed over a Sephadex G-25 column, using 0.1 M acetic acid as solvent, and the eluted peptides were lyophilized. The peptides were then subjected to chromatography on columns of carboxymethylcellulose (15) or on columns of hydroxylapatite (16). Fractions obtained by these procedures were then subjected to gel filtration on Bio-Rad agarose, A-5m, in 6 M urea-0.05 M Tris-HCl, pH 7.5. Finally, both analytical and preparative SDS-acrylamide gel electrophoresis was carried out (17, 18). The identity of the cross-links in hydrolyzed peptides was monitored by using the amino acid analyzer and locating the cross-links by their radioactivity (11).

Analytical Procedures—Ultraviolet spectra from 600 to 200 nm were recorded on a Cary model 14. Gel filtration was carried out on a calibrated column (0.9 cm × 60 cm) of Bio-Gel P-2 developed with 0.1 M acetic acid at room temperature. Periodate oxidation was done according to Bohak (19) except that the volumes were scaled down 10-fold.

Esterification was carried out by the conventional HCl-alcohol method except that the samples were refluxed in glass tubes (13 mm × 100 mm) which were capped with Teflon-lined screw caps and were heated at 80°C in a dry-block test tube heater for 1 to 2 hours. The reaction mixture was taken to dryness by means of a nitrogen stream and the sample was placed in a vacuum over KOH pellets overnight. Trifluoroacetic anhydride was then added and the capped sample was stored at room temperature for 6 hours after which the anhydride was evaporated in a stream of nitrogen. The acetylation and permethylation were accomplished by standard methods (20). Derivatized compounds were analyzed in a Hitachi RMU-6E spectrometer, using perfluorokyl ethers (Peninsular Chemicals) as internal standards. High resolution mass spectra were obtained on an AEI MS-902 instrument.

Detection of imidazole compounds by sequential reduction and diazotization was carried out as described (21). NMR spectra were obtained on samples dissolved at approximately 10⁻³ M in D₂O, using sodium 3-trimethylsilylpropionate-2,2,3,3-d⁴ as an internal standard. The measured pH was between 6.8 and 7.0 in all cases and was not corrected for a deuterium isotope effect. The spectra were recorded at a probe temperature of ~22°C on a Varian XL-100 instrument (courtesy of Dr. George Gray, Varian Associates), using the pulsed-wave, Fourier transform technique. Generally, 1000 pulses were accumulated in order to obtain a satisfactory signal to noise ratio.

RESULTS

The isolation of the two compounds whose chemical properties are described in detail below was accomplished by sequential ion exchange chromatography. The initial step was to fractionate the protein hydrolysate using Dowex 50-X8, eluting with the potassium formate and potassium acetate buffers. The radioactive profile obtained from this preparative chromatography resembled the analytical profiles obtained when a small portion of the hydrolysate was chromatographed on the amino acid analyzer (3-8). Those radioactive peaks which appeared to be new substances were individually pooled and then titrated with 60% HClO₄ at 4°C until the precipitation of KClO₄ ceased; the precipitate was removed by centrifugation. The supernatant fluid was concentrated by rotary evaporation and subjected to chromatography on Bio-Rad Aminex A-5 resin (as described under "Experimental Procedures"). Generally, one major and several minor radioactive peaks were detected; a portion of the major peak was then analyzed for its content of amino acids using the amino acid analyzer with ninhydrin detection. At this point, substantial purity (>50%) of the unknowns was often noted, and final purification was effected by the Aminex A-5 column, by modifying the gradients. Alternatively, the sample was applied to the amino acid analyzer column and the effluent was collected in fractions, omitting the ninhydrin stream. An entire prepara-

1 The abbreviations used are: SDS, sodium dodecyl sulfate; TFA, trifluoroacetyl.
Chromatographic elution times of histidine-containing cross-links

System A: 0.9 X 13 cm; Spinco PA-35 resin; 0.35 M sodium citrate, pH 5.25, 80 ml per hour. System B: 0.9 X 55 cm; Spinco UR-30 resin; 0.25 M sodium citrate, pH 2.9; complex gradient to 0.4 M citric acid; 80 ml per hour. System C: 0.9 X 21 cm; Bio-Rad Aminex A-5 resin; 0.2 M pyridine acetate, pH 3.5; linear gradient to 0.8 M pyridine acetate, pH 5.2; 60 ml per hour; 100 ml in each gradient chamber. All columns were run at 50°C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chromatographic elution time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>System A</td>
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<tr>
<td>Aldol-histidine</td>
<td>17</td>
</tr>
<tr>
<td>Histidino-hydroxymerodesmosine I</td>
<td>48</td>
</tr>
<tr>
<td>Histidino-hydroxymerodesmosine II</td>
<td>50</td>
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</table>

Table I

Aldol-histidine was a white fluffy powder, freely soluble in water, and it stained brown when sprayed with iodine, but did not react with the Pauly reagent (21). It eluted as a single, symmetrical peak on the amino acid analyzer (Table I); this peak was identical with the location of the tritium radioactivity when the column effluent was measured. The ultraviolet spectrum of the compound (in H2O) was unremarkable, both at acid and alkaline pH; it showed a typical "end-absorption" curve. The NMR spectrum (Fig. 1A) showed characteristic singlets at 7.71 ppm (C-2, imidazole) and 7.08 ppm (C-4, imidazole), a characteristic triplet centered at 4.00 ppm (α-CH, histidine), and a characteristic doublet (δ-CH2, histidine) at 3.20 and 3.14 ppm. The envelope centered about the spike at 3.76 ppm was attributed to the non-histidine α-CH groups and to a single CH2OD group. The envelope about the spike at 1.96 ppm was attributed to a single methine group plus the protons from two β-CH2 groups, while the envelope centered at 1.32 ppm was attributed to the protons of two γ-CH2 groups plus one δ-CH2 group. Finally, the proton of the methine group attached to the imidazole nitrogen is thought to be located in the envelope associated with the triplet at 4.00 ppm. All of these assignments are based upon values in the literature (29) and upon our comparative studies using methyl-1-histidine and methyl-3-histidine as references. These studies showed that the protons of C-4 (imidazole) at 7.02 ppm and those of the methyl group at 3.72 ppm were at the same chemical shifts for both methyl histidines. In contrast, however, the other protons differed significantly in their chemical shifts in both methyl histidines. Thus, for methyl-1-histidine, the C-2 (imidazole) proton was at 7.85 ppm, the α-CH proton at 3.92 ppm, and the β-CH2 protons centered at 3.22 ppm. For methyl-3-histidine these same protons were at 7.67 ppm, 3.98 ppm, and 3.11 ppm, respectively. Therefore, the NMR spectrum of aldol-histidine is consistent with an N-alkyl histidine which is substituted at the N-3 position rather than at the N-1 position.

The mass spectrum of the trifluoroacetyl methyl ester derivative of aldol-histidine showed a molecular ion of 855, consistent with an elemental composition of C32H30O8N2F12. As detailed previously (13), this composition was confirmed by high resolution mass spectrometry and was further established by determining the composition of several fragments derived from the molecular ion. Of particular note was the imidazole fragment at m/e 266, which represents the histidino portion of the mole-
cule plus 2 protons (13). Also of importance was the related ion peak at m/e 206 which was attributed to the 296 peak minus the ester fragment (COOCH₃) and a proton. Finally, the mass spectrum (Fig. 2) of the acetylated, permethylated derivative of aldol-histidine was entirely consistent with the other data. Fig. 2 shows the anticipated molecular ion of 653 and the characteristic fragments, resulting from simple cleavages, which were present at m/e 638, 622, 610, 594, 582, 567, 551, 537, 523, and 509. The important fragments at m/e 467 and 423 correspond to benzylic cleavages at the C-6 branch point, similar to those seen in the mass spectrum of the TFA methyl ester derivative (13). The imidazole portion of the structure was represented by the fragments at m/e 230, 225, 210, 104, and 166, consistent with the earlier results (13). Thus, the most likely structure of aldol-histidine is that shown in Fig. 3; it can be seen that, in addition to the three α-carbon groups, two other potential sites of stereoisomerism are present. Our data do not distinguish which form(s) have been isolated, nor is it absolutely certain which of the ring nitrogens is alkylated although both model building and the NMR data suggest that it is N-3 as shown.

General Properties of Histidino-hydroxymerodesmosine—The preparative work-up yielded a single radioactive substance, which was slightly asymmetrical in the pyridine acetate ion exchange system but which appeared as a broad, symmetrical peak in the amino acid analyzer (Fig. 4). It was found to co-chromatograph with the “post-histidine” peak seen in hydrolysates of borohydride reduced, reconstituted collagen fibrils (12). When spotted on filter paper, it appeared light blue under ultraviolet light and it stained brown when sprayed with iodine, but it did not react with the Pauly reagent (21). Under certain chromatographic conditions, histidino-hydroxymerodesmosine resolved into two components (Fig. 5 and Table I). Each of these components had identical chemical properties in all of the studies reported here.

The compound, when subjected to heating in 6 M HCl or 2 M NH₄OH at 107° for varying lengths of time, was stable to acid but was slowly destroyed by ammonia (70% remaining at 48 hours). Exposure to sodium metaperiodate at room temperature rapidly destroyed histidino-hydroxymerodesmosine, yielding three ninhydrin-positive products which eluted later than the original compound (Fig. 4). Approximately 35% of the original ninhydrin color yield, expressed as leucine equivalents, was present in the three peaks. Most of the tritium was found in the area of ammonia when the column effluent was collected. Reduction with NaBH₄ after the periodate oxidation did not change the distribution of ninhydrin-reactive or labeled products. The parent compound, prior to oxidation, gave a strong positive

![Fig. 3](image-url) Postulated structure of the amino acid, aldol-histidine (2,10-diamino-5-hydroxymethyl-6-(N-3-histidyl)-undecanedioic acid).
test for tertiary-quaternary amines in the iodoplatinate test when compared with standard compounds (24).

The ultraviolet spectrum of the compound was unremarkable, at pH 1, pH 7, and pH 11, showing an "end-absorption" curve.

Comparison of the specific activity of this compound with the other cross-links obtained from the same collagen hydrolysates (Table II) indicated that histidino-hydroxymerodesmosine had about twice as many ninhydrin-reactive groups, assuming that all of the compounds had incorporated the same amount of tritium. Comparison of histidino-hydroxymerodesmosine and reduced desmosine by gel filtration suggested that they were of similar size.

The NMR spectrum of histidino-hydroxymerodesmosine (Fig. 1B) was, in some respects, similar to that of aldol-histidine, particularly regarding the histidyl protons. It also showed: (a) an increase in the number of methylene protons appearing at 1.0 to 2.3 ppm; (b) an increase in the α-CH signals centered at 3.76, compared to the α-CH triplet of histidine (4.0); (c) a new doublet at 2.85 ppm, attributed to a methylene group adjacent to a secondary amine; (d) another doublet at 3.02, attributed to an n-CH2 adjacent to a secondary amine. All of the specific features related to histidine, namely, the imidazole singlets at 7.71 and 7.08, the α-CH triplet centered at 4.0, and the β-CH2 doublets centered about 3.17 were present as was the signal attributed to the methine-imidazole proton at 4.2. The broad peak at 4.2 also contains the signal from the methine of the secondary alcohol group. Thus, the NMR spectra suggested that aldol-histidine and histidino-hydroxymerodesmosine were related compounds although they were widely separated during ion exchange chromatography (Table I).

The mass spectrum of the trifluoroacetyl methyl ester derivative of NaBDd-reduced histidino-hydroxymerodesmosine (Fig. 6) showed a molecular ion of 1266, consistent with an elemental composition of C40H44D01~N~F1~. The presence of one deuterium atom in the compound was confirmed by examining the trifluoroacetyl methyl ester derivative of NaBHe-reduced histidino-hydroxymerodesmosine, which had a molecular ion of 1205 (the other high mass fragments were also one atomic mass unit less than those seen in Fig. 6). The presence of four carboxyl groups in histidino-hydroxymerodesmosine was established by the "shift" technique, comparing the mass spectra of the trifluoroacetyl methyl and ethyl ester derivatives. Thus the latter derivative had a molecular ion of 1261, compared to m/e 1205 for the TFA methyl ester derivative, showing that four carboxyl groups are present. Similarly, the total number of acylated groups was established by comparing the TFA methyl ester derivative and the pentfluoropropionyl methyl ester derivative (Fig. 7). The largest ion detected in the latter spectrum was at m/e 1386 (M – C2F5O), apparently arising from a molecular ion of 1505. The ions at 1341 (M – 164; loss of C2F5COOCH) and 1282 (M – (164 + 59); loss of C2F5COOCH and CO2CH2) corroborated this interpretation. (The ions at 1268 and 1237 probably arose from the presence of incompletely esterified derivatives.) Exact mass measurements (Table III) showed that the 1341, 1282, and 1268 fragments had elemental compositions which supported the origins attributed to these ions. Thus, it seemed that six groups became acylated in this compound under the conditions employed.

Interpretation of the mass spectrum in Fig. 6 was aided by comparison with that of the trifluoroacetyl methyl ester derivatives of aldol-histidine (13) and other collagen cross-links (10). Virtually all of the higher mass fragments and most of the prominent fragments could be accounted for by well known losses. Thus, the simple cleavages of M – F (1187); M – OCH2 (1175); M – CO2CH2 (1147) were present, as was the ion due to the loss of trifluoroacetate, M – CF3COOH (1092). This latter loss, in conjunction with simple cleavages, accounted for the ions at 1077 (M – (CF3COOH + CH2)); 1061 (M – (CF3COOH + OCH2)); 1033 (M – (CF3COOH + CO2CH2)); 1023 (M – (CF3COOH + CF2)); 995 (M – (CF3COOH + CF2CO)) and 985 (M – (CF3COOH + CF3CONHCH2CO2H)). This latter ion most likely resulted from benzylic cleavage between the α and β carbon of the histidyl moiety, as noted in the

### Table II

<table>
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<th>Compound</th>
<th>Specific activity</th>
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<tr>
<td>Histidino-hydroxymerodesmosine</td>
<td>Batch 1*: 3.7 × 10^5 ppm/umole</td>
</tr>
<tr>
<td></td>
<td>Batch 2: 2.3 × 10^5 ppm/umole</td>
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<td></td>
<td>Batch 3: 0.83 × 10^5 ppm/umole</td>
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<tr>
<td>Hydroxylysinonorleucine</td>
<td>8.9 × 10^5 ppm/umole</td>
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<tr>
<td>Dihydroxylysinonorleucine</td>
<td>2.2 × 10^5 ppm/umole</td>
</tr>
<tr>
<td>Lysinonorleucine</td>
<td>7.8 × 10^6 ppm/umole</td>
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</table>

* Each batch was prepared separately with different lot numbers of NaBH3.

* Leucine equivalents.
case of aldol-histidine. Furthermore, the ions at 980 ($M - 226$) and 504 ($M - 702$) also correspond to benzylic cleavages about the methine which is attached to the imidazole nitrogen. Branching of one of these benzylic side chains (702) is suggested by the presence of the two complementary ions at 728 and 478, the latter ion corresponding to $N$-$\epsilon$-methylene, $N$-$\epsilon$-TFA, $O$-$\epsilon$-TFA, $N$-$\alpha$-TFA hydroxylysine methyl ester. Other ions can be related to the cyclic loss of $N$-trifluoroacetyl $O$-trifluoroacetyl hydroxypipeolic acid methyl ester (351). This loss, in conjunction with other losses, accounts for the ions at 835 ($M - (351 + HF)$) and 796 ($M - (351 + CO_2CH_3)$). The branch which gives rise to these cyclic losses probably is due to a $\delta$-hydroxylysine moiety because in other spectra (not shown) an ion at 968 ($M - 338$) was occasionally detected. (The loss of 338 would result from cleavage between the $\delta$ and $\epsilon$ carbons of a hydroxylysyl moiety.) Assignment of some of these ions was corroborated by exact mass measurements (Table III) of the trifluoroacetyl methyl ester of NaBH₄-reduced histidino-hydroxymerodesmosine.

![Figure 7. Mass spectrum of the pentafluoropropanoyl methyl ester derivative of NaBH₄-reduced histidino-hydroxymerodesmosine.](image)

**Table III**

<table>
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<tr>
<th>Derivative</th>
<th>m/e</th>
<th>Observed mass</th>
<th>Calculated mass</th>
<th>Composition</th>
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<td>1091</td>
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<td>1032</td>
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<td>1032.2624</td>
<td>C₁₀H₁₄N₆O₁₂F₁₅</td>
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<tr>
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<td>503</td>
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<td>503.1365</td>
<td>C₁₀H₁₂N₆O₁₂F₁₅</td>
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<td></td>
<td>475</td>
<td>475.0515</td>
<td>475.0525</td>
<td>C₁₀H₁₀N₆O₁₂F₁₅</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>265.0695</td>
<td>265.0674</td>
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<tr>
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<td>Pentafluoropropionyl methyl ester</td>
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<td></td>
<td>1268</td>
<td>1268.238</td>
<td>1268.231</td>
<td>C₁₀H₁₄N₄O₁₂F₁₅</td>
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</table>

![Figure 8. Partial mass spectra of the trifluoroacetyl methyl ester (A) and ethyl ester (B) of NaBH₄-reduced histidino-hydroxymerodesmosine.](image)

**Figure 9.** Postulated structure of the amino acid, histidino-hydroxymerodesmosine (2,10-diamino-5-(5-hydroxylysinoorn-leucyl)-3-(N-3-histidyl)-undecanioic acid).
The imidazole fragments (Fig. 8) were as prominent in the spectrum of trifluoroacetyl methyl ester of histidino-hydroxymerodesmosine as those seen in the equivalent spectrum of aldol-histidine. As anticipated, the methyl and ethyl ester imidazole fragments differed in mass only by the contribution of the ester group (Fig. 8). (Compare \( m/e \) 280 (ethyl ester) and 266 (methyl ester) versus the common ion \( m/e \) 206 for both esters.) Thus, all of the evidence concerning the structure (Fig. 9) of histidino-hydroxymerodesmosine is consistent and supports its relationship to aldol-histidine. Similar to aldol-histidine, histidino-hydroxymerodesmosine potentially has a number of stereoisomers. Thus, in addition to the four \( \alpha \)-carbon groups there are three potential sites of stereoisoromerism, and
conceivably, either of the two imidazole ring nitrogens may be substituted. The chromatographic separation of two peaks (Fig. 5 and Table I) which have identical chemical properties probably reflects some form of isomerism; epimerization of the CHOH moiety during acid hydrolysis, as occurs in the case of hydroxylysine, is a strong possibility. As noted in the case of aldol-histidine, the NMR data and model building support the N-3 nitrogen as the probable alkyl attachment site.

![Figure 14](image)

**Figure 14.** Gel filtration on Bio-Rad A-5m of the second peak of Fig. 12. Conditions were: 1.5 × 95 cm; 200 to 400 mesh Bio-Rad agarose A-5m; 6 M urea in 0.05 M Tris-HCl, pH 7.5; 1 ml per fraction; 12 ml per hour; 25 mg sample in 1 ml applied to column. Horizontal bars indicate pooled fractions.

![Figure 15](image)

**Figure 15.** Diagram of the SDS-acrylamide gel electrophoresis of the pooled fractions from Fig. 14. Conditions are described in Reference 17. The location of collagen marker peptides is noted. Radioactivity was determined by slicing the stained bands, dissolving them in 30% H₂O₂ and adding liquid scintillation fluid (1). An unstained slice served as control.

**Fig. 16.** Ion exchange chromatography of an acid hydrolysate of NaBH₄-reduced rabbit corneal and scleral collagen fibrils. Conditions were those of System B in Table I, collecting 2 ml per fraction.

![Figure 16](image)

**Fig. 17.** Ion exchange chromatography of an acid hydrolysate of NaBH₄-reduced reconstituted and natural cow skin collagen fibrils. Conditions were those of System A in Table I, collecting 1 ml per fraction.
Peptides Containing Aldol-histidine and Histidino-hydroxymerodesmosine—Chromatography of the CNBr digest of NaBH₄-reduced collagens on CM-cellulose columns showed that, despite a clear-cut elution pattern of peptides as determined by absorbance (Fig. 10), the profile of radioactivity was quite spread out (Fig. 11). Examination of the radioactive effluent showed that the relative abundance (percentage of total counts per min) of both aldol-histidine and histidino-hydroxymerodesmosine increased as one progressed through the chromatogram. The combined effluent from the latter part of the chromatogram (fractions 110 to 160) was applied to a column of hydroxylapatite and eluted as shown in Fig. 12. The second peak had a specific activity three to five times greater than the original material applied to the column and about 10 times greater than the first peak. Moreover, both histidine-containing cross-links were present mainly in the second peak (Fig. 13), whereas the first peak consisted mostly of reduced aldehydes and reduced, bifunctional cross-links (Fig. 13).

The second peak from the hydroxylapatite column was passed through a gel filtration column (Fig. 14) and the fractions were pooled as shown. These fractions were subjected to SDS-acrylamide gel electrophoresis which showed that several large radioactive peptides were present in the mixture (Fig. 15).

Abundance of Aldol-histidine and Histidino-hydroxymerodesmosine—Since these two compounds are quite unusual in structure, a measure of their relative abundance, compared to other NaBH₄-reducible collagen cross-links was made. Surveys of various tissues showed that, whereas histidino-hydroxymerodesmosine was quite prevalent (Fig. 16), aldol-histidine was present but was abundant only in cow skin collagen (Fig. 17). Assuming that both compounds may be reduced to the same extent in vivo, it is not clear why there is such a disparity in their relative abundance.

**DISCUSSION**

The isolation and characterization of these new, histidine-containing, tritium-labeled, polyfunctional amino acids from NaBH₄-reduced collagens is the first demonstration that: (a) histidine participates in collagen cross-linking, and (b) that larger compounds, capable of cross-linking several polypeptide chains, are present in collagen. Contrary to expectation, the structure of these polyfunctional compounds is quite different from the pyridinium cross-links of elastin. However, they are related to such pyridinium compounds in the sense that they may arise from similar precursor molecules. For example, unreduced aldol-histidine can be conceived of as deriving from a Michael addition of the imidazole of histidine to the α-carbon of the α,β unsaturated bond of an aldol condensation product (Fig. 18). This latter substance is present in both collagen and elastin. Since an active carbonyl moiety remains in unreduced aldol-histidine, Schiff base formation can ensue, yielding unreduced histidino-hydroxymerodesmosine (Fig. 18). Alternatively, dehydrohydroxymerodesmosine may form initially, similar to the dehydromerodesmosine observed in elastin (25, 26) and then Michael addition may occur. That this latter pathway is also operative is suggested by the finding that small quantities of a compound which has a mass spectral pattern consistent with hydroxymerodesmosine can be isolated from sodium borohydride-reduced insoluble collagen.

The participation of histidine in collagen cross-linking is particularly noteworthy because only small amounts of histidine (less than 1% of the total amino acids) are present in collagen (27, 28) and the histidines appear to be very specifically distributed (28). In contrast, α-amino adipic acid-δ-semialdehyde may be more extensively distributed throughout collagen although this seems to vary with the collagenous tissue or the method of extraction and purification (29, 30). However, the aldol condensation product of this aldehyde seems to occur at only one locus (29, 30), and kinetic studies show it to be consumed as histidino-hydroxymerodesmosine (12). This observation, in conjunction with the limited locations of the few histidines, supports the concept that collagen intermolecular cross-linking is controlled by stereospecific interactions of adjacent tropocollagen molecules in the fibrous polymer (1).
The contribution of collagen histidine to these new compounds is clearly shown by using reconstituted fibrils as the source of reducible cross-links; no other histidine contribution is possible when purified tropocollagen is used to form reconstituted fibrils. Ancillary evidence is furnished by the isolation from collagen of partially purified CNBr peptides which contain the histidine-derived cross-links; similar peptides were present in the digests of both naturally insoluble and reconstituted collagen fibrils. The size range of these purified peptides is that anticipated from the combination of those peptide(s) which contain the aldol condensation product and those peptides which contain histidine, especially those from the α2 chain of collagen.

The usefulness of hydroxylapatite in specifically fractionating the peptides containing polyfunctional cross-links probably has its basis in two well described phenomena. First, Bernardi has shown that fully denatured proteins do not adhere to hydroxylapatite, although tropocollagen will adhere in 1 M urea. Under such conditions, virtually none of the applied peptides adhere to the column. Thus, the addition of hydroxylapatite to the existing methods for studying collagen structure should provide a convenient aid in obtaining purified, cross-linked collagen peptides in 1 M urea will probably be partially denatured, but those peptides containing cross-links, particularly the larger peptides, might be expected to be in the most native-type conformation. Consequently, such peptides may preferentially adhere to hydroxylapatite, as observed. To reinforce this line of reasoning, we have carried out experiments identical with those in Fig. 12, only substituting 6 M urea for 1 M urea. Under such conditions, virtually none of the applied peptides adhered to the column. Thus, the addition of hydroxylapatite chromatography to the existing methods for studying collagen structure should provide a convenient aid in obtaining purified, cross-link-containing peptides. Hopefully, the availability of such peptides will ultimately shed light on the packing of tropocollagen molecules in the fibrous, polymeric network.

These new histidine-containing amino acids, by virtue of their multiple amino and carboxyl groups, can potentially unite several polypeptide chains, both those within a tropocollagen molecule and those between adjacent molecules. Thus, they may serve to form a continuous polymeric network, accounting in part for the insolubility of the bulk of collagen in many tissues.

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