A stable nonreducible trifunctional cross-linking amino acid has been isolated from mature bovine skin collagen fibrils. Previous cross-link peptide isolations and amino acid analyses indicate the compound has properties identical with those of hydroxyaldolhistidine. Its newly proposed structure was verified using fast atom bombardment mass spectrometry, and $^1$H and $^{13}$C nuclear magnetic resonance. The data indicated that the cross-link consists of the prosthetic groups from one residue each of histidine, hydroxylysine, and lysine. The $^1$H and $^{13}$C nuclear magnetic resonance data indicated that imidazole C-2 of histidine is linked to C-6 of norleucine ($^\epsilon$-deaminated lysine residue) in turn linked to the C-6 amino group of hydroxylysine. Based on the trivial names for other cross-linking residues found in collagen and elastin it was given the name histidinohydroxylysinonorleucine. In vitro incubation studies for up to 24 weeks, in aqueous solution at physiological pH and ionic strength, using 6-month-old bovine embryo skin demonstrated a one-to-one stoichiometric relationship between the disappearance of the labile reducible bifunctional cross-link dehydrohydroxylysinonorleucine and the appearance of histidinohydroxylysinonorleucine. These results can partially explain the previously observed disappearance of dehydrohydroxylysinonorleucine with chronological age.

The covalent intermolecular cross-links between molecules in collagen fibrils are essential in providing connective tissue matrices with their stability, cohesiveness, and physicochemical properties. Cross-linking in type I collagen fibrils is initiated by enzymatic oxidative deamination of $\epsilon$-amino groups to aldehydes of specific peptidyl residues of lysine and hydroxylysine located in COOH- and NH$_2$-terminal nonhelical regions of the collagen molecule, with conversion starting at physiological pH and ionic strength, using 6-month-old bovine embryo skin demonstrated a one-to-one stoichiometric relationship between the disappearance of the labile reducible bifunctional cross-link dehydrohydroxylysinonorleucine and the appearance of histidinohydroxylysinonorleucine. These results can partially explain the previously observed disappearance of dehydrohydroxylysinonorleucine with chronological age.

Structure and Formation of a Stable Histidine-based Trifunctional Cross-link in Skin Collagen*

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A stable nonreducible trifunctional cross-linking amino acid has been isolated from mature bovine skin collagen fibrils. Previous cross-link peptide isolations and amino acid analyses indicate the compound has properties identical with those of hydroxyaldolhistidine. Its newly proposed structure was verified using fast atom bombardment mass spectrometry, and $^1$H and $^{13}$C nuclear magnetic resonance. The data indicated that the cross-link consists of the prosthetic groups from one residue each of histidine, hydroxylysine, and lysine. The $^1$H and $^{13}$C nuclear magnetic resonance data indicated that imidazole C-2 of histidine is linked to C-6 of norleucine ($^\epsilon$-deaminated lysine residue) in turn linked to the C-6 amino group of hydroxylysine. Based on the trivial names for other cross-linking residues found in collagen and elastin it was given the name histidinohydroxylysinonorleucine. In vitro incubation studies for up to 24 weeks, in aqueous solution at physiological pH and ionic strength, using 6-month-old bovine embryo skin demonstrated a one-to-one stoichiometric relationship between the disappearance of the labile reducible bifunctional cross-link dehydrohydroxylysinonorleucine and the appearance of histidinohydroxylysinonorleucine. These results can partially explain the previously observed disappearance of dehydrohydroxylysinonorleucine with chronological age.

The covalent intermolecular cross-links between molecules in collagen fibrils are essential in providing connective tissue matrices with their stability, cohesiveness, and physicochemical properties. Cross-linking in type I collagen fibrils is initiated by enzymatic oxidative deamination of $\epsilon$-amino groups to aldehydes of specific peptidyl residues of lysine and hydroxylysine located in COOH- and NH$_2$-terminal nonhelical regions of the collagen molecule, with conversion starting in the COOH-terminal peptide (1).

Recently it has been shown that both peptidyl Lys-16' and Hyl-16' in the COOH-terminal nonhelical peptides of the third amino acid, histidine, involved in the formation of this cross-link has now been identified as residue number 92 in the $\alpha_2$(I) chain and is the subject of another report (24). The quantitative significance of this cross-link and its distribution in different tissues and among different species is not yet known.

Location of deH-HLN 1, in bovine periodontal ligament collagen (2) as well as in young bovine skin (3), along with the locus of trifunctional cross-link in mature bovine skin (4, 24) demonstrated that the latter encompasses the same molecular positions as the former cross-link. We, therefore, hypothesized a possible precursor-product relationship between the reducible iminium cross-link deH-HLN1 and the stable non-

1 The abbreviations used are: HAH, hydroxyaldolhistidine; deH-HLN 1, dehydrodehydrolysinonorleucine, HLN, hydroxylysinonorleucine; HHL, histidinohydroxylysinonorleucine; FAB, fast atom bombardment; HPLC, high performance liquid chromatography; TES, 2-[tris(hydroxymethyl)(methyl)amino]-ethanesulfonic acid; COSY, correlated spectroscopy.

2 M. Yamauchi and G. L. Mechanic, unpublished results.
reducible trifunctional cross-link. We report here in vitro incubation studies of skin that strongly support this putative precursor-product relationship. Based on this information we, therefore, re-evaluated the structure of this potentially important skin collagen cross-link. We also propose a possible mechanism for its formation.

The identical chromatographic behavior (two different systems) of HAH (obtained from Dr. Housley, University of Connecticut, by Dr. Kuboki, Hokkaido University, Japan and M. Y.), the isolated cross-linking residue from skin collagen (reported here) and from the isolated three-chained peptides (4, 21, 24), indicates that HAH (3) is equivalent to the compound whose structure and formation is the subject of this report.

**MATERIALS AND METHODS**

**Isolation of the Cross-linking Compound**—Approximately 100 g of the dermal layer from 2.5-year-old bovine skin was diced and hydrolyzed by refluxing with 1.5 liters of 6 N HCl at 105 °C for 48 h. The hydrolysate was filtered, evaporated to dryness under reduced pressure at 40 °C, and dissolved in 100 ml of distilled water. This solution (approximately 10 ml each time) was then applied to a phosphocellulose column (P11, H⁺ form, 2.5 × 15 cm, equilibrated with 0.1 N HCl) by a method modified from that described previously by Fuji-moto (5). Approximately 1 liter of 0.1 N HCl was pumped through the column at a flow rate of 150 ml/h at room temperature to remove the bulk of the amino acids. A linear gradient from 0.1 to 1 N HCl was then employed with a total volume of 500 ml. Fractions of 10 ml were collected, and 0.2 ml of each fraction was subjected to ninhydrin assay. The ninhydrin-positive fractions which eluted at 0.6-0.8 N HCl were pooled and evaporated to dryness. The pooled fractions were then rechromatographed on a previously standardized column (1 × 100 cm) of Bio-Gel P-2 (~400 mesh) that had been equilibrated with 20% acetic acid (2). Two-ml fractions were collected at a flow rate of 5 ml/h at room temperature with UV monitoring at 230 nm. An example of a typical chromatographic profile of reduced and nonreducible collagen cross-linking compounds is depicted in Fig. 1. The major peak eluted at the position of standard cross-linking compound (previously identified as HAH) prepared from a hydrolysate of the isolated cross-linked peptides (4, 21, 24). The compound was finally purified using a Varian 5560 HPLC equipped with a stainless steel amino acid column (AA911, Interaction) (2) and a fraction collector. One-ml fractions were collected, and 0.01 ml of each fraction was subjected to ninhydrin assay. The major ninhydrin positive peak which appeared at the elution position of the standard cross-linking compound (4, 24) was pooled. The purified component was desalted on a Bio-Gel P-2 (1 × 100 cm, ~400 mesh) column equilibrated with 0.5% acetic acid and then lyophilized.

**Amino Acid Analysis**—The samples were assessed for their amino acids using a Varian 5560 HPLC configured as an amino acid analyzer using ninhydrin, with color development at 135 °C in a stainless steel reaction coil (2). A cross-link standard of HLNL was prepared from NaBH₄ reduced skin collagen hydrolysate. An apparently pure three-chained cross-link peptide containing the cross-link was prepared from unreduced mature bovine skin collagen by the methods described previously (4, 21, 24). The hydrolysate of this peptide and the HLNL standard were used to identify the elution positions on the amino acid analyzer and obtain the color factor for the trifunctional cross-link (see below). Analyses were performed in duplicate.

**Fast Atom Bombardment Mass Spectrometry**—Mass spectrometric analyses were performed on a VG ZAB-4F four-sector instrument of B1E1-E2B2 configuration (VG Analytical, Manchester, United Kingdom) (6). Ions were generated by fast atom bombardment using a xenon primary beam of 8 keV energy. Approximately 20 µg of sample was applied to the matrix consisting of a 5:1 mixture of dithiothreitol/dithioerythritol. The resolution of MS1 (B1E1) was about 1000 (10% valley definition). Helium was used as the collision gas in the cell located in the third field-free region (between E1 and E2) at a pressure corresponding to a 50% diminution of the parent ion beam. The collision energy was 8 keV, and the resolution of MS2 (E2B2) was unit or better. Daughter ion spectra of ions selected by MS1 were monitored by linked-field scanning of B2 and E2, at constant B2/E2 ratio. The data were acquired on a VG 11/250 data system using a procedure described recently (7) to calibrate MS2.

**Identifying H and 13C NMR Spectra**—One-dimensional 1H NMR spectra were recorded at ambient temperature on a Bruker WM-250 spectrometer, locked on the D₂O solvent. Additional spectral assignments were based on a two-dimensional COSY experiment (8, 9). 13C NMR spectra were obtained at 75.48 MHz on a GE QE-300 NMR spectrometer on 6.4 mg of the cross-linking compound dissolved in 0.4 ml of H₂O/0.1 ml of D₂O, with the latter serving as a lock. The 20% D₂O solution was used in order to minimize any possible exchange of the histidine imidazole C-2 proton (if present in the structure) with the deuterium from the solvent. A broad hump is the 13C spectrum, which is a feature of the 1H/13C probe for the QE-300 supplied by General

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**Fig. 1.** Composite Bio-Gel P-2 (400 mesh chromatographic column profile for standard cross-links from a complete acid hydrolysate of [1H]NaBH₄-reduced and unreduced collagen. Absorbance (closed circles with solid line) was read at 570 nm after ninhydrin color development. Radioactivity (open circles with broken line) represents the cpm in 0.02 ml of each fraction. Pyridinoline (PYR) elutes after bifunctional cross-links in this system probably due to its aromaticity. HHL elutes between the tetrafunctional histidine hydroxymenedesmosine (HHMD) and bifunctional cross-links dihydroxylysino- }

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Electric NMR, was removed using a base-line correction program.  

**Incubation Studies**—Skin from a 6-month-old bovine embryo was thoroughly cleaned of hair and fat with a scalpel in the cold. This was then pulverized at liquid N₂ temperature using a freezer mill (Spex, Metuchen, NJ), washed with 0.02 M sodium phosphate buffer, pH 7.4, deionized H₂O, and lyophilized. Approximately 20-30 mg samples of the prepared skin were suspended in separate vials each containing 0.15 M TES buffer at pH 7.4, 0.001 M β-aminopropionitrile, and a drop of toluene as a bacteriostatic agent. The samples were then sealed and incubated at 37°C. Samples were removed from the incubator at various periods of time and reduced with standardized [³H]NaBH₄ by the method described previously (2).  

Specific Activity of [³H]NaBH₄—The specific activity of the [³H] NaBH₄ was determined in the same manner as described previously (2). As apparently pure peptide containing dihydroxylysinoanorleucine isolated from reduced dentin collagen was used in this instance. The results indicated that the specific activity of [³H]NaBH₄ was 3.47 × 10⁷ dpm/μmol.  

Cross-link Analyses—[³H]NaBH₄-reduced cross-links were analyzed by the method described previously (2). deH-HNLNL was detected as its reduced product HNLNL. HHL and hydroxyproline were determined by amino acid analysis using ninhydrin. Prior to the determination of HHL, an aliquot of the complete collagen hydrolysate from each incubation sample of known hydroxyproline content was applied to the standardized P-2 column described above (equilibrated with 0.6% acetic acid) to remove the bulk of amino acids as well as other ninhydrin-positive substances. The fractions encompassing the elution position of standard HHL from each were pooled, lyophilized, and subjected to amino acid analysis. The content of the cross-links was expressed in mol/mol of collagen based on a value of 300 residues of hydroxyproline/molecule of collagen. The ninhydrin color factor for HHL was determined using the residue composition of the apparently pure three-chain peptide containing the cross-link (4, 24). It was 1.97 times more than leucine.  

**RESULTS**  

**Amino Acid Analysis**—The isolated cross-linking compound was judged to be more than 99% pure based on amino acid analyses employing two different gradient systems. The chromatographic profile using a gradient system for enhanced resolution (2) is shown in Fig. 2. The yield of the compound was 7.0 mg. The compound had the identical chromatographic properties as that of the cross-link from the isolated three-chain peptides (4, 21, 24) and that of the HAH supplied by Housley (see the Introduction) using two different gradient systems.  

**Fast Atom Bombardment Mass Spectrometry**—The source spectrum shows a protonated molecular ion (MH⁺) at m/z 445, thus indicating a molecular weight of 444 for the compound. The spectrum does not provide total information about the complete structure since a number of eventual fragments of interest are obscured by the intense matrix background. However, this problem is obviated by use of the daughter ion spectrum of 445, since only the fragmentations of the MS₁-selected parent ion are monitored. Such a spectrum is presented in Fig. 3, which represents the average of 10 consecutive scans. The majority of the prominent fragments can be directly related to the proposed structure by the following cleavages. The ions at m/z = 428, 427, and 370 result from the loss of NH₃, H₂O, and HOOC-CH₃-NH₂ from the MH⁺, respectively. The signals at m/z = 342, 312, 300, and 283 arise by loss of HOOC-CH(NH₂)-CH₂-CH₃, HOOC-CH(NH₂)-(CH₂)₄-OH, HOOC-CH(NH₂)-(CH₂)₂-CH(OH)-CH₃, and HOOC-CH(NH₂)-(CH₂)₂-CH(OH)-CH₂-NH₂, respectively, all from the hydroxylysine moiety. Fragments at m/z = 237, 182, 128, and 110 correspond to a consecutive loss of HCOOH, HOOC-CH(NH₂)-CH₂-CH₃ histidine and histidine + HCOOH, respectively, from m/z = 283. It must be noted that we also observed these four ions in the daughter ion spectrum of m/z = 283. Finally, the signals appearing at m/z = 163 and 156 account for the protonated hydroxylysine and the protonated histidine, respectively. m/z = 156 is also the base peak in the daughter ion spectrum of m/z = 283. Therefore, the spectrum in Fig. 3 provides strong confirmatory evidence for the structure depicted in Fig. 6.  

Additional evidence that the spectrum is concordant with the proposed structure is obtained from other ions. The ions at m/z = 342, 312, 300, and 283 indicate that the hydroxylysine moiety is attached through the 6-amino group to the rest of the molecule. It can be noted that the signal at m/z = 342 can arise from two different parts of the molecule as shown in the figure. Ions at m/z = 163 and 156 indicate that hydroxylsine and histidine are linked by one bond to the lysine moiety. At this time, we do not have a full explanation for some ions such as m/z = 400, 370, 329, 209, and 169. They cannot result from simple cleavage of MH⁺ unless a violation of the even-electron rule with the formation of odd-electron (radical) neutral fragments has occurred. This is highly unlikely and, in addition, has never been reported for this type...
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FIG. 3. Daughter ion spectrum of the protonated cross-linking compound generated by fast atom bombardment. The parent ion at 445 is off scale by a factor of about 100, and the upper trace has been magnified by a factor of 5. The major cleavages are shown on the proposed structure.

cf molecule. However, the latter observed ions are probably due to rearrangements that occur during FAB. Our FAB analysis deals with an underivatized and protonated molecular ion. The mass spectrometry performed previously was with a completely derivatized product which led to completely different fragmentation pathways (3). Therefore, the results obtained here cannot be directly compared with the previous structural analysis of this trifunctional cross-link; however, they strongly confirm and are consistent with the structure depicted in Fig. 6.

$^1H$ and $^{13}C$ NMR Spectra—The aromatic region of the $^1H$ NMR spectrum contains only a single uncoupled resonance at 7.11 ppm relative to tetramethylsilane. Based on studies of L-histidine (10), this may be assigned to the imidazole C-4 proton of histidine (Fig. 4). This spectrum differs from that previously obtained by Housley et al. (3) which exhibited two aromatic resonances. Based on their structure, these resonances were assigned to the olefinic proton of the putative enamine double bond in their proposed structure and to the two histidyl ring protons. This led them to conclude that the linkage to histidine was via the nitrogen atom (N-3) of the imidazole ring. In order to eliminate the possibility that the imidazole C-2 proton had exchanged with deuterium from the solvent, a series of $^1H$ spectra was obtained at 5 min, 1 h, and 15 h after dissolution of the cross-link in $D_2O$. There was no evidence of time-dependent spectral changes, and even at 5 min no C-2 proton resonance was observed (Fig. 4). As expected, the two-dimensional COSY experiment reflects the couplings within the amino acid components of the cross-link but does not provide direct information on the nature of the cross-link since in each case the protons from the component amino acids are separated by more than three bonds and hence do not exhibit observable coupling. However, the observation of only a single lysine H-6 proton triplet (due to coupling with H-5 and 5') is consistent with the proposed structure. Assigned resonance shifts in the $^1H$ NMR spectrum are: lysine: H-2, 3.8 ppm; H-3 and 3', 1.9, 2.0 ppm; H-4 and 4', 1.5, 1.6 ppm; H-5, 3.9 ppm; H-6 and 6', 2.7, 3.0 ppm; histidine: H-2, 4.0 ppm; H-3 and 3', 3.2 ppm; imidazole ring H-4, 7.1 ppm.

The $^{13}C$ NMR spectrum (Fig. 5) provides additional strong and clear evidence to support the proposed structure (Fig. 6) confirmed by the FAB spectra. The $^{13}C$ shifts of the cross-link have been compared with those for histidine and lysine derived from the literature (11, 12) as well as with that of 5-
(OH) lysine which was calculated from the lysine shifts based on the expected OH group substituent effects (13). The results are summarized in Table I. From this comparison, it can be seen that with the exception of the three resonances corresponding to lysine C-6, histidine imidazole carbons C-2 and C-5, the agreement is quite good. The cross-link clearly exhibits no resonance near 40 ppm, the shift of the unsubstituted lysine C-6. As a model for the effect of the histidine linkage on the shift of the lysine C-6 carbon, one can compare the carbon-13 shift of alanine C-3 with that for histidine C-3, indicating a chemical shift perturbation of 12 ppm (11, 12). This is in reasonable agreement with the assignment of lysine C-6 to the group of resonances at 54.8 ppm. The effect of the linkage on the imidazole C-2 of the histidine resonance can be modeled, for example, by a comparison of the shift of the C-4,5 resonance of imidazole (122.3 ppm) (14) with that of histidine (128.4 ppm) suggesting a 5.9-ppm downfield shift upon substitution. This leads to a predicted ring C-2 shift of 135.0 ± 5.9 - 140.9 ppm, which is in good agreement with the observed shift of 141.8 ppm. There is additionally a downfield shift of the quaternary C-5 resonance of histidine upon the formation of the cross-link. Further support for this interpretation comes from an examination of the intensities of the histidine imidazole ring carbon resonances, suggesting that both of the downfield-shifted carbons are quaternary and hence partially saturated under the pulsing conditions used in the study (Fig. 5).

In summary, the $^{13}$C NMR spectrum obtained is consistent with the presence of the three amino acids, lysine, hydroxylysine, and histidine, as the sole amino acids that constitute this stable nonreducible trifunctional cross-link. There are relatively few chemical shift perturbations relative to the free amino acids, but these are consistent with the linkage model shown in Fig. 6. Furthermore, the intensity of the imidazole C-2 resonance of histidine which is similar to that of C-5 and less than half that of C-4 is consistent with its conversion to a quaternary carbon. Similar spectral characteristics were observed for the substituted C-2 in 2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine (diphthamide) (22). The assignments given above are also consistent with attached proton (Spin Echo Fourier Transform) spectra (15) which we obtained. Based on the proposed structure verified by FAB mass spectrometric and NMR data an obvious structural designation is HHL (Fig. 6). As discussed below, this cross-link might be formed as a consequence of the condensation of a histidine residue with the bifunctional iminium cross-link deH-HLNL.

**Incubation Studies**—The changes in the content of the two cross-links, deH-HLNL and HHL, upon incubation of intact skin collagen at 37°C are shown in Fig. 7. The content of the cross-links is expressed in mol/mol of collagen based on a value of 300 residues of hydroxyproline/molecule of collagen.  

| Incubation Period (Weeks) | Moles/Mole of Collagen |
|--------------------------|------------------------|
| 0                        | 0                      |
| 4                        | 0.1                    |
| 8                        | 0.2                    |
| 12                       | 0.3                    |
| 16                       | 0.4                    |
| 24                       | 0.5                    |

A stable nonreducible trifunctional cross-linking amino acid was isolated from mature bovine skin collagen. The

**DISCUSSION**
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compound was chromatographically identical (two gradient systems) to the compound HAH (supplied by Housley et al.) found in collagen (3), in a chromatographic peak of collagen-derived tryptic three-chained peptides (3, 21), and in apparently homogeneous peptides purified from the latter mixture (4, 24). Its structure was identified as histidinohydroxylysinosinonorleucine using fast atom bombardment mass spectrometry, \(^1\)H NMR, and \(^{13}\)C NMR. The structural elucidation indicates that the imidazole C-2 of histidine is linked to C-6 on the norleucine (€-deaminated lysine) portion of the molecule which in turn is linked to the \(\epsilon\)-amino group of hydroxylysine. This structure is different from the one proposed by Housley et al. (3) which was HAH and was suggested to form by the putative condensation of 5-amino-5-carboxypentanal, 2-hydroxy-5-amino-5-carboxypentanal, and imidazole N-3 of histidine \((M_r = 427)\). Their data were based on conventional mass spectrometry and the fragmentation pattern obtained from the trifluoroacetylated methyl ester derivative of the cross-link. The parent peak they observed had \(M_r = 853\) whereas the fully derivatized HHL would be 1062. Their observed parent peak was possibly due to a secondary parent which resulted from a loss of 209 in the neutral moieties CF₃CONH₂ and three CH₃OH groups. The odd \(M_r\) of 853 indicates an odd number of nitrogen atoms and would then be consistent with loss of an N from a fully derivatized HHL in the form of a neutral CF₃CONH₂, as suggested above to perhaps yield a parent of an odd \(M_r\). In any event, in order to agree with the \(^1\)H NMR data (peak at 7.65 ppm) Housley et al. (3) obtained, they proposed a structure for HAH which embodied an enamine type configuration. It must be noted that they should have obtained a significant peak at about 8.5 ppm for the H atom of C-2 in their structure which would have been more consistent with the \(^1\)H NMR spectrum of histidine (22). Perhaps the compound Housley et al. (3) subjected to \(^1\)H NMR was slightly contaminated.

Previously we attempted to specifically cleave the trifunctional cross-link "HAH" (3) at its putative double bond with OsO₄/NaIO₄ (23) while in peptide form (4). Double bonds are reported to cleave quantitatively with the latter reagent (23). An anomalous unknown partial degradation occurred and, as a result, unexpectedly low yields of cleavage products were obtained. However, the correct identities for the two peptide chains, linked by a newly chemically produced unknown bifunctional cross-link, were obtained. The reaction products of the cleaved cross-link were not identified, and, therefore, premature confirmation of the structure proposed by Housley et al. (3) for HAH was made (4).

In this report it is seen that the molecular weight of the cross-link obtained by FAB is 444. The even \(M_r\) indicates it possesses an even number of nitrogen atoms. The latter two facts and its fragmentation pattern confirm the structure proposed for HHL (Fig. 6). The configuration is consistent with a condensation of deH-HLNL and histidine. In addition, \(^1\)H NMR (one-dimensional spectra at 5 min, 1 h, and 15 h after dissolving the cross-link in D₂O), two-dimensional COSY, as well as \(^{13}\)C NMR further confirmed the proposed structure seen in Fig. 6 and established the linkage position of the imidazole C-2 of histidine to deH-HLNL. The previously proposed structure for the cross-link involved an imidazole N-3 link which would demand protons on C-2 and C-4 of the imidazole ring. However, in this report we find the imidazole C-2 is linked to a carbon atom. The possibility that the C-2 proton had exchanged with deuterium from the solvent and, therefore, would not be observed has been ruled out (see results of \(^1\)H and \(^{13}\)C NMR). The structure (Fig. 6) clearly indicates a possible precursor-product relationship between deH-HLNL and HHL. The incubation studies (4-24 weeks) using skin collagen tend to substantiate this type of relationship (Fig. 7) because stoichiometry was observed between the loss of the iminium cross-link deH-HLNL and the formation of the stable nonreducible trifunctional cross-link HHL.

In the past, a number of investigators have reported and

\[
\begin{align*}
\text{(I)} & & \text{(II)} & & \text{(III)} \\
\begin{array}{c}
\text{H} \\
\text{OH}
\end{array} & & \begin{array}{c}
\text{H} \\
\text{OH}
\end{array} & & \begin{array}{c}
\text{H} \\
\text{OH}
\end{array} \\
\text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2 & \longrightarrow & \text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2 & \longrightarrow & \text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2\text{C}^\circ\text{N}^\circ\text{CH}_2
\end{align*}
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\[
\begin{align*}
\text{(IV)} & & \text{(V)} & & \text{(VI)} \\
\text{H} & & \text{H} & & \text{H} \\
\text{O} & & \text{O} & & \text{O} \\
\text{H} & & \text{H} & & \text{H} \\
\text{N} & & \text{N} & & \text{N} \\
\text{H} & & \text{H} & & \text{H} \\
\text{C} & & \text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C} & & \text{H}_2\text{C} \\
\text{C} & & \text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C} & & \text{H}_2\text{C} \\
\text{C} & & \text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C} & & \text{H}_2\text{C}
\end{align*}
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\begin{align*}
\text{(VII)} & & \text{(VIII)} \\
\text{H} & & \text{H} \\
\text{O} & & \text{O} \\
\text{H} & & \text{H} \\
\text{N} & & \text{N} \\
\text{H} & & \text{H} \\
\text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C} \\
\text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C} \\
\text{C} & & \text{C} \\
\text{H}_2\text{C} & & \text{H}_2\text{C}
\end{align*}
\]

**FIG. 8.** Postulated mechanism of formation of histidinohydroxylysinosinonorleucine. See "Discussion" for details.
proposed mechanisms for the disappearance of deH-HLNL from collagen with chronological aging of the organism (16, 17). This was based on the disappearance of HLNL after reduction with \([\text{[H}]\text{borohydride and subsequent analysis of the acid-hydrolyzed reduced collagen. In addition, both Robins et al. (16) and Davis et al. (17) observed its disappearance upon \textit{in vitro} incubation of intact native collagen fibrils, as we have found in this report. In order to account for the disappearance of deH-HLNL, the latter authors (17) suggested further condensations with free \(\epsilon\text{-NH}_2\) groups of hydroxylysine and lysine residues by the latter groups’ nucleophilic attack on the unsaturated carbon atom of the iminium compound deH-HLNL to form saturated gem-diamines and/or carbinol amines as end products of the reaction. The latter mechanisms are clearly untenable since both types of compounds are highly labile, are only transitory in nature and are unstable to acid hydrolysis (18). In addition, reduction with borohydride causes the latter two classes of compounds to revert back to their original products, in which case the original amounts of HLNL would have been recovered (18).

More recently Bailey et al. (19) isolated some \(\alpha\)-aminoacids and acid from both collagen and elastin and suggested, in the case of collagen, that the carbonyl involved in the iminium compound deH-HLNL is oxidized to a carboxyl group to form a peptide bond between the \(\epsilon\text{-NH}_2\) of hydroxylysine and the newly formed \(\delta\text{-COOH}\) group of the \(\alpha\)-aminoacidic acid. This mechanism is still unproven since no two-clained peptides, as yet, have been isolated from collagen or elastin which contain an \(\alpha\)-aminoacidic acid \(\delta\text{-COOH}\) to \(\epsilon\text{-NH}_2\) peptide bond.

A postulated mechanism for the formation of HHL is presented in Fig. 8. The carbonionium (II) produced by protonation of the iminium nitrogen of deH-HLNL (I) is stabilized by the resonance structure (III) by charge delocalization. Then electrophilic substitution at C-2 (20) of the imidazole of histidine residue (IV) by the carbonionium ion (II) occurs to yield the transition state compound (VII) which is stabilized by resonance structures (V and VI). The loss of a proton from imidazole C-2 to some proton acceptor leads to the restoration of the stable aromatic structure (VIII) complete formation of the cross-link HHL.

Quantitative studies using a variety of whole aged skin collagen from bovine as well as human sources indicated that HHL was the major age-related cross-link and demonstrated a continuous increase with chronological aging in both species.

The possibility that HHL is an artifactual product in skin collagen fibrils is ruled out, since we have already demonstrated the isolation of apparently homogeneous tryptic peptides containing this cross-link and identified the molecular location of the specific hydroxylysine, lysine (4), and histidine (24) residues which are involved in its formation.

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