A Natural Histidine-based Imminium Cross-link in Collagen and Its Location*

Paul H. Bernstein‡ and Gerald L. Mechanic§

From the Dental Research Center and Department of Biochemistry and Nutrition, University of North Carolina, Chapel Hill, North Carolina 27514

The purpose of this report was to investigate by two independent means the status of the tetrafunctional cross-link dehydrohistidinohydroxymerodesmosine in collagen fibrils and to locate in the protein molecule the contributing histidine residue. These aims are consistent with obtaining insights into the three-dimensional packing of collagen molecules in fibrils. Insoluble collagen fibrils treated at pH 4.3 to break the cross-link into its precursors were re-equilibrated at pH 7.4, 37°C, to allow re-formation of dehydrohistidinohydroxymerodesmosine. It was demonstrated that there was a time-dependent increase in its re-formation which reached control values at 24 h. The data were consistent with the Michael addition of the N of histidine across the ethylene double bond of the α,β unsaturated aldehyde, aldol condensation product, the interchain intramolecular cross-link of collagen.

Iodination of purified reconstituted soluble guinea pig skin collagen fibrils at pH 7.4 destroyed all but 1 to 2 residue sites/mmol of collagen with full retention of dehydrohistidinohydroxymerodesmosine. This indicated that the N of the histidine residue participating in the cross-link was protected from modification by alkylation as the Michael adduct. Pepsin solubilization of the iodinated fibrils, denaturation, gel filtration, and O-(carboxymethyl)cellulose chromatography allowed isolation of two α1 chains. Chromatographic differences were probably due to fractional site participation of imidazole with iodine. Preparative polyacrylamide gel electrophoresis of CNBr digests of the two α1 chains isolated from the modified fibrils allowed preparation of α1CB5-8 and α1CB7,6 from each. These CNBr peptides contain the histidines present in α1. Amino acid analyses indicated 1 residue of histidine in each of α1CB5-8 peptides and a fractional residue in each preparation of α1CB7,6. It was surmised, therefore, that histidine 89 of the α1 chain participates in the formation of histidinohydroxymerodesmosine obtained from NaBH₄-reduced collagen fibrils. It was concluded from the reformation study and the iodine modification work that dehydrohistidinohydroxymerodesmosine is a real and natural cross-link in collagen fibrils and not an artifactual compound catalyzed by the borohydride or cyanoborohydride anions in the reduction procedure as claimed by Robins and Bailey (Robins, S. P., and Bailey, A. J. (1973) Biochem. J. 135, 657-665; Robins, S. P., and Bailey, A. J. (1977) Biochem. J. 163, 339-346).

In vitro reconstituted soluble collagen fibrils are stabilized by imminium intermolecular covalent cross-links. These are reducible with NaBH₄ to secondary amines (Tanzer and Mechanic, 1968). The same phenomena occur with natural macromolecular collagen fibrillar matrices (Mechanic and Tanzer, 1970). Many of the reducible cross-links have been isolated and identified as their reduced products (Tanzer, 1973). The intermolecular cross-links are synthesized by condensation of specific peptidyl aldehydes with ε-amino groups of specific peptidyl-Lys and Hyl residues. The aldehydes are derived from Lys and Hyl residues by oxidative deamination of the ε-amino groups by lysyl oxidase. Lys yields α-aminoacidic-δ-semialdehyde and Hyl yields δ-hydroxy-ε-aminoacidic-δ-semialdehyde. In guinea pig skin collagen, peptidyl-ε-aminoacidic-δ-semialdehyde occurs in the NH₂- and COOH-terminal nonhelical peptides of α1 chains (Fukae and Mechanic, 1980). Intramolecular, interchain cross-links form when 2 residues of α-aminoacidic-δ-semialdehyde in terminal nonhelical peptides of two α chains combine in an aldol-type condensation to form aldol (an aldol condensation product containing an α,β unsaturated aldehyde), thereby producing a β chain (Bernstein et al., 1966; Bernstein and Pies, 1966).

Franzblau et al. (1970) demonstrated that reduced aldol and the “Post His” Peak in cross-link profiles of [³H]NaBH₄-reduced reconstituted collagen fibrils bore a precursor-product relationship, respectively. The “Post His” cross-link later was demonstrated to be the polyfunctional compound His-OHMerDes' (Tanzer et al., 1973). These authors proposed that its formation occurred by Michael addition of the N' of His to the β carbon of the α,β unsaturated aldehyde, (aldol) and then condensation of the resulting new aldehyde, aldol-His, with the ε-amino of Hyl to form the imminium compound dehydro-His-OHMerDes. Reduction with NaBH₄ converted the Schiff base to the secondary amine, His-OHMerDes, which also resulted in stabilizing the cross-link.

The hypothesis that dehydro-His-OHMerDes is a natural cross-link in collagen was brought into question by Robins and Bailey (1973) and (1977). They claimed their experimental evidence indicated that the polyfunctional cross-links aldol-His and dehydro-His-OHMerDes did not exist in vivo. These authors put forth the hypothesis that upon reduction with NaBH₄ (Robins and Bailey, 1973) and NaBH₄CN (Robins and Bailey, 1973) the imminium compound dehydro-His-OHMerDes was not reduced at the ε-amino group of His.

The abbreviations used are: His-OHMerDes, histidinohydroxymerodesmosine; dehydro-His-OHMerDes, unreduced precursor of His-OHMerDes, dehydrohistidinohydroxymerodesmosine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; CM-cellulose, O-(carboxymethyl) cellulose.

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‡ Present address, Department of Oral Biology, University of Connecticut Health Center.

§ To whom all communication should be addressed.

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Bailey, 1977), the formation of reduced aldol-His and His-OHMerDes are products of base-catalyzed condensations caused by the reducing agents anhydrides (BH₂ and BH₂CN⁻) and, as a result, the compounds were artifacts of the reduction procedure.

More recently, a His-containing cross-link hydroxyaldol-His has been isolated from unreduced insoluble steer skin (Hous-ley et al., 1975). Only 14 of approximately 3150 amino acid OHMerDes are products of base-catalyzed condensations, has been isolated from unreduced insoluble steer skin collagen, and, as a result, the compounds were artifacts of the reduction that stabilize collagen matrices. In studying structure-functional relationships of collagen, our laboratory has been interested in the location and distribution of those amino acid residues that contribute their prosthetic groups to the various intermolecular cross-links in order to obtain insights into the three-dimensional packing of collagen molecules in fibrils.

Experiments in our laboratory with insoluble steer skin collagen using an extension of the Robins and Bailey (1973, 1977) method has led us to believe that dehydro-His-OHMerDes is a natural cross-link in collagen fibrils.

It is known that when the imidazole ring of His is protonated, it is not susceptible to commonly used modification techniques. Westhead (1972) demonstrated that protonated imidazole ring was resistant to photo-oxidation. Resistance to alkalization by protonated His-imidazole was shown by Glazer et al. (1975) while Wolff and Covelli (1969) reported that alkylated by protonated His-imidazole was resistant to photo-oxidation or iodination. This report documents the use for $1.05 per set of photocopies. Full sized photocopies are available from the Journal of Biological.

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Natural Cross-links in Collagen

Fig. 1. Time-dependent formation of dehydrohistidinohydroxymerodesmosine in collagen upon re-equilibration of insoluble fibrils at pH 7.4, 37°C, that had been pretreated with phosphate buffer, pH 4.3, for 4 h at room temperature. Dehydrohistidinohydroxymerodesmosine was detected as [3H]histidinohydroxymerodesmosine (HHMD) after reduction of the fibrils with [3H]NaBH₄.

Fig. 2. Per cent of His modified in insoluble collagen fibrils by iodination. The fibrils were pretreated with phosphate buffer pH 4.3 for 4 h and then incubated in pH 7.4 phosphate buffer at 37°C for 36 h. Portions at each time point were taken and treated with iodine.

at pH 4.3 and then re-equilibrated at pH 7.4 (37°C) for various periods of time is shown in Fig. 1. The data indicate a gradual increase in the formation of dehydro-His-OHMerDes. By 24 h, it was found that the reduced re-equilibrated fibrils contained 64,150 cpm of [3H]His-OHMerDes/μmol of Hyp which represented quantitative reformation of the cross-link.

Fig. 1 indicates that the formation of dehydro-His-OHMerDes is a kinetic phenomenon that requires time, much like any organic chemical reaction involving a Michael addition.

Iodination of Re-equilibrated Insoluble Collagen Fibrils—Insoluble collagen fibrils subjected to pH 4.3 phosphate buffer for 4 h and then allowed to re-equilibrate at pH 7.4 for various time periods were iodinated at 0°C. It was found that the sample subjected to iodination immediately after being brought to pH 7.4 lost more than 90% of its His and by 1/2 h had lost 92%. As time progressed at pH 7.4 (Fig. 2), iodination modified somewhat less His and a minimum was reached at 24 h where about 17 to 22% of the His was recovered on amino acid analysis. This is consistent with the time-dependent formation of dehydro-His-OHMerDes. The early data points clearly indicate that His residues in acidified collagen fibrils unprotonate almost immediately upon being raised to pH 7.4. It was therefore concluded that pH equilibration within collagen fibrils is very rapid.

Iodination of Soluble Collagen Fibrils—Studies on the iodination of collagen at constant reaction time (4 h) using varying concentrations of I₂ and at constant concentration of...
Natural Cross-links in Collagen

Figure 5

a) Gel filtration of denatured purified soluble collagen on AcA 34 column (Bio-bead swelled).

b) Gel filtration of denatured purified iodinated recombinant soluble collagen fibrils. Peak 3 corresponds to chain, peak 4 eluting last prior to iodinated Heps are terminal peptides cleaved from collagen chains by papain.

Figure 6

I, for varying times are presented in Fig. 3, a and b. Fig. 3a shows that His content had a sharp decrease from 0.05 mM to 0.455 mM I, and then a gradual decrease to 2.5 mM (Fig. 3a). Using a constant concentration of 2.5 mM, it was readily ascertained that 2 h (Fig. 3b) were sufficient essentially to complete modification of the susceptible His residues in collagen fibrils.

Based upon the above finding, the large batch of collagen fibrils was iodinated (see "Experimental Procedures" for details).

Electron microscopic examination and measurements of the band spacings in the iodinated fibrils indicated a normal banding pattern.

Amino acid analysis of the aliquot of control unreduced unmodified collagen fibrils contained values for the amino acid composition that were in agreement with the published literature (Clark and Bornstein, 1972; Gallop and Faz, 1975).
The value for the ratio Phe/His was 2.13. The NaBH₄-reduced unmodified fibrils had a ratio of Phe/His of 2.46, indicating the loss of about 13% His, representing 1 residue/mol of collagen. Analysis of a portion of the iodinated fibrils indicated a Phe/His ratio of 12.32 which represented a loss of 87 to 88% His or recovery of 1 to 1.5 His residues/mol of collagen.

Base hydrolysis of the control collagen reduced as molecules demonstrated the presence of 37,800 dpm of aldol/mol of Hyp which indicated 1.03 mol of aldol/mol of collagen.

A chromatogram of the [³H]NaBH₄-reduced unmodified fibrils is shown in Fig. 4a. Rechromatography of the His-OHMerDes yielded apparently pure cross-link, as shown in Fig. 4b. Summation of the radioactivity indicated 35110 dpm His-OHMerDes/µmol of Hyp which represents a value of 0.94 mol of collagen. Rechromatography of the hydroxylsiononorleucine peak also yielded pure material. A chromatogram of [³H]NaBH₄-reduced iodinated fibrils is depicted in Fig. 5a. The putative hydroxylsiononorleucine peak on rechromatography indicated the presence of an unknown compound. There was an 86% loss of hydroxylsiononorleucine. Rechromatography of the peak eluting just prior to His-OHMerDes, as seen in Fig. 5a, indicated the presence of His-OHMerDes and an unknown compound (Fig. 5b), while the

**TABLE I**

Amino acid analysis of control and modified collagen and isolated chains

<table>
<thead>
<tr>
<th>Actual values listed for less than 10 residues.</th>
<th>Unmodified control</th>
<th>Peptated control</th>
<th>Peptated, isolated fibrils</th>
<th>Peptated, isolated fibrils</th>
<th>A-5m nmol</th>
<th>a-1 chain fraction</th>
<th>Peak III</th>
<th>CM-52 Peak 3</th>
<th>CM-52 Peak 7</th>
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</table>

*Uncorrected for destruction.

**TABLE II**

Amino acid composition of peptides obtained from α₁ chains that were obtained from unmodified collagen fibrils

<p>| Values are expressed as residues per peptide, actual values listed for residues present as less than 10 residues. Value of 0 none was found. |</p>
<table>
<thead>
<tr>
<th>Peak 3 (Fig. 10)</th>
<th>Peak 7 (Fig. 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1CB5-8</td>
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<td>a1CB7.6</td>
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<td>Lys</td>
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<td>Arg</td>
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</table>

* Uncorrected for destruction on hydrolysis.

**FIG. 10.** Mechanisms of formation of imminium compounds and their reduction by borohydride or cyanoborohydride anion. Taken from March (1966) and Borch et al. (1971). The reduction of the imminium cation in the center takes place by hydroxide ion addition as long as a source of H· ions are present. This reduction can take place with either BH₃CN⁻ or BH₄⁻ at neutral pH. At acid pH (3 to 4), the imminium compound is unstable and reductive alkylation takes place with BH₃CN⁻ because of the stability of the reducing reagent in acid solution. BH₄⁻ hydrolyzes faster at acid pH, than it can transfer a hydroxide ion to a reducible group.

**FAST**

His-OHMerDes (Fraction 339 to 343) (Fig. 5a) was apparently pure (Fig. 5c). Integration of the radioactivity of His-OHMerDes indicated a total of 35,500 dpm/µmol of Hyp which represents 0.95 mol/µmol of collagen. It was clear that although only 1 to 1.5 residues of His were left/µmol of collagen after iodination, no loss of the cross-link His-OHMerDes occurred.

**Locus of the Unmodified His in Unreduced Iodinated Fibrils**—It was found that, after iodination, the collagen fibrils were sparingly soluble in dilute acetic acid solution. Partial peptic digestion of native collagen has the ability of bringing insoluble collagen into solution and only cleaves the terminal nonhelical peptide regions without attacking the helical por-
tion (Rubin et al., 1965; Drake et al., 1966; Kuhn et al., 1966; and Weiss, 1975). This method was used to solubilize the collagen, a portion of which was titrated to pH 7.4 in the cold; the clear viscous solution was reduced with the standardized [3H]NaBH4. Analyses after acid and base hydrolysis indicated conservation of the same amount of aldol was present as in unmodified collagen reduced as molecules. No hydroxylysino norleucine nor His-OHMerDes was found. The only radioactive peaks depicted in Fig. 6b were obtained with the gradient and, after elution of the treated control collagen. The PAGE pattern of the corresponding collagen samples depicted in Fig. 6, a and b, are shown in Fig. 7, a and c. Note that pepsin treatment increased the proportion of a chains as seen in Fig. 7, b and c. (See figure legends for details.)

The PAGE patterns of the three major peaks after gel filtration of pepsin-treated iodinated fibrils (Fig. 6b) are shown in Fig. 8. Most of the material in peak 3 (Fig. 6b) was shown by PAGE to consist mainly of a chains (Fig. 8c). Peak 3 from the Bio-Gel A-5m column was further purified on columns of CM-Cellulose (see "Experimental Procedures" for details) whose elution profile is presented in Fig. 9. Six major peaks were obtained with the gradient and, after elution of the column with 0.01 M LiOH, 0.2 M LiCl upon completion of the gradient, another proteinaceous peak emerged (Peak 7).

PAGE demonstrated that Peaks 3 and 7 were better than 95% homogenous and corresponded to a1 chains. Amino acid analysis indicated no Tyr and only 1 residue of His in each (Table I). Except for His and Tyr, the compositions were very similar to an a1 chain (Clark and Bornstein, 1972).

It was apparent after PAGE that all the other peaks contained a2 chain and in no case was it less than 50% contained by a1. Rechromatography was unsuccessful in preparing a2 chain in pure enough form for analyses.

Amino acid analyses of the column volume peak (Peak 4) of pepsin-solubilized, iodinated fibrils (Fig. 6b) indicated the presence of Tyr and the complete absence of His. This suggested that the His involved with the cross-link dehydro-His-OHMerDes was retained with the modified collagen chains after peptic cleavage. The presence of Tyr suggested that Peak 4, Fig. 6b contains material from the terminal ends of the collagen molecule, as would be expected.

CNBr Cleavage of Isolated a1 Chains—Low initial yields of CNBr peptides were obtained from purified a1 chains isolated from iodinated collagen fibrils. Treatment of the a1 chains with 1% mercaptoacetic acid led to more complete cleavage with CNBr, as determined by PAGE. The patterns obtained were identical with those obtained recently (Fukae and Mechanic, 1980). His only occurs at positions 89, 942, and 103 in the a1 chain, the first being in a1CB5 and the latter two as constituents of a1CB7,6. Preparative PAGE was therefore used to prepare the CNBr peptides a1CB5-8 and a1CB7,6 from Peaks 3 and 7 (Fig. 9). Incomplete cleavage of the a1CB5-8 occurs with CNBr (Fukae and Mechanic, 1980), and no Met exists between a1CB7 and a1CB6 in guinea pig skin collagen. Amino acid analyses of the two CNBr peptides (Table II) derived from Peak 3 indicated 1.0 residue of His in a1CB5-8 and 0.2 residues in a1CB7,6. Analyses of the peptides (Table II) obtained from Peak 7 indicated the presence of 1.1 residues of His in a1CB5-8 and 0.2 residue in a1CB7,6. Except for the fractional residues of His in both preparations of a1CB7,6 the compositions were almost identical with those published previously (Clark and Bornstein, 1972). In addition, the constituents of both a1CB5-8 peptides isolated were the same as the combined compositions of a1CB5 and a1CB8 previously reported (Clark and Bornstein, 1972). Conservation of the one His in a1CB5-8 from two chromatographically separable a1 chains from iodinated collagen fibrils strongly suggest that this His was protected during the modification reaction. Chromatographic heterogeneity of a1 chains was probably due to partial site participation in the iodination reaction. This has been noted previously with modification reactions of proteins (Crestfield et al., 1963).

**DISCUSSION**

In this report we demonstrate that reformation of the cross-link dehydro-His-OHMerDes is a time-dependent reaction (24 h) in insoluble collagen fibrils after it has been cleaved at acid pH (Fig. 1). The reaction is slow compared to reduction with NaBH4 or NaBH4CN (Borch et al., 1971) and is concordant with a Michael condensation which is the mechanism proposed by Tanzer et al. (1973). The time dependency is not uncommon, for Mitra and Lawton (1979) report complete Michael addition of 2-p-nitrophenyl) allyl-4-nitro-3-carboxy-

The numbering system of the amino acid residues in an a1 chain used is that of Pietzack and Kuhn (1976) where 1-104 represents the sequence in the NH2-terminal nonhelical peptide region, 1-1014 represents the sequence in the helical peptide region, and 1-5-255 represents the sequence in the COOH-terminal nonhelical peptide region.
phenyl to ribonuclease takes 36 h and the reagent's addition to ε-amino groups of polylysine by Michael addition takes 22 h. Robins and Bailey (1977) also found that the Michael addition, at pH 4.4, of hydride, from NaBH₄CN, to the β carbon of aldol in the formation of dihydrohydroxymerodesmosine, took 24 h to reach maximal amounts.

Aqueous moderate thermal conditions, as well as dilute acid treatment of collagen fibrils, destroys dehydro-His-OHMerDes (Bailey, 1968; Bailey et al., 1970; Robins and Bailey, 1973; Jackson et al., 1974; Robins and Bailey, 1977). The Michael adduct imminium cross-link almost certainly undergoes retro-Michael (retrograde Michael) reaction. There are many documented cases in the literature for the occurrence of retro-Michael reactions (Patai and Rappoport 1962, 1962a, 1962b, 1962c; Patai et al., 1962; Grunbaum et al., 1966; Shenhav et al., 1970; Burckhalter and Brown, 1950). Retro-Michaels occur in acetic acid solution (Suzuki and Sekiya, 1976) or at moderate thermal conditions (Allen, 1966) or in hot water at moderate temperatures (Allen et al., 1964; Allen and Happ, 1964). Furthermore, the Michael adduct of 2-(p-nitrophenol) allyl-4-nitro-3-carboxyphenyl and ribonucleosine spontaneously undergoes a series of retro-Michael reactions (Mitra and Lawton, 1979). Mitra and Lawton (1979) also report that the foregoing Michael adduct is stabilized by reduction of the electron-withdrawing group preventing the occurrence of retro-Michael reaction. The same phenomena occur when reduction of the electron-withdrawing groups, carboxyl (aldol-His) and imminium (dehydro-His-OHMerDes), is accomplished with NaBH₄. The reduced Michael adduct between 2-(p-nitrophenol) allyl-4-nitro-3-carboxyphenyl and the ε-amino of Lys was isolated from the 6 N HCl hydrolysate of the poly(Lys) reacted material, indicating it was stable to hydrolysis, whereas the unreduced Michael adduct underwent retro-Michael upon acid hydrolysis.

Data from experiments reported by Robins and Bailey (1973, 1977) led them to doubt that dehydro-His-OHMerDes was a natural in vivo crosslink in collagen. They believed that formation of the cross-link His-OHMerDes in reduced collagen fibrils was artificial and was caused via catalysis by the anions of the reducing reagents, BH₄⁻ (Robins and Bailey, 1973) and BH₄⁺CN⁻ (Robins and Bailey, 1977). However, the data in this report does not allow such an interpretation. Furthermore, we contend that the data obtained by the latter authors conformed with the chemical reactions involved when collagen is reduced under nonphysiological conditions. Robins and Bailey (1973, 1977) maintain that dehydro-His-OHMerDes does not exist in collagen fibrils at pH 7.4 and 8.9 but that the imminium cross-link dehydrohydroxymerodesmosine does. If this were the case, they should have obtained small amounts of the artificial compound, dihydrohydroxymerodesmosine, upon reduction with NaBH₄CN at pH 7.4 and 8.0 (Robins and Bailey, 1977), and they only found His-OHMerDes. Michael addition of hydride from NaBH₄CN occurs with α,β unsaturated carbonyl-type compounds (Borch et al., 1971) and would have competed with imidazole for reaction with dehydrohydroxymerodesmosine, which did not occur. Moreover, formation of His-OHMerDes using NaBH₄CN was completed in 1 h leading Robins and Bailey (1977) to propose that cyanoborohydride anion is an efficient catalyst for Michael addition of imidazole to dehydrohydroxymerodesmosine. However, as described above, Michael addition is a relatively slow reaction when compared with the fast reduction of iminium compounds by borohydride or cyanoborohydride (Borch et al., 1971) (Fig. 10), and hydroxymersdeosine should have resulted instead, but did not. We believe that Michael addition of hydride was precluded because of the already existing cross-link dehydro-His-OHMerDes, as our data indicate. Thus, His-OHMerDes was formed in a relatively short period of time. It is our belief that dehydrohydroxymerodesmosine does not exist in collagen fibrils, and the most plausible explanation for its non-existence and the existence of dehydro-His-OHMerDes agree with the simple principles involved in the chemistry of carbonyl compounds. The α,β unsaturated aldehyde is a stable compound and, according to the method of Berthier and Serre (1966), exists and reacts as depicted.

\[
H \quad R' \quad H \quad H' \quad R \quad C \quad C \quad C \quad C \quad O
\]

\[
\begin{align*}
H' & = R' = H \\
R & = C = C = O
\end{align*}
\]

The newly formed aldehyde, aldol-His, then reacts as depicted in Fig. 10, as would an ordinary aldehyde, to form the imminium compound dehydro-His-OHMerDes, or it can be reduced by borohydride to form reduced aldol-His as was reported by Fairweather et al. (1972). Apparently, dihydrohydroxymerodesmosine (Robins and Bailey, 1977) results from NaBH₄CN reduction of collagen at pH 4.4 to 6.0 by reductive alkylation of the ε-amino of hydroxylysine by the Michael adduct of hydride and aldol. These reactions are known to occur with carbonyl and amine compounds in the presence of NaBH₄CN at acid pH (Borch et al., 1971). The same is true in the formation of hydroxylsylsinosonorleucine under the same conditions as found by Robins and Bailey (1977). It is quite possible that although the collagen fibrils were at acid pH, which is known to disrupt imminium bonds, the insoluble fibers maintain estamido aldehyde in close juxtaposition to the ε-amino of hydroxylysine so that reductive alkylation can occur.

Robins and Bailey (1973) reported finding equal amounts of hydroxylsylsinosonorleucine when collagen fibrils in 0.9% NaCl solution at pH 4.3 and 7.4 were treated with an equal volume of 0.001 M NaOH containing [1H]KBH₄. We found that there was an instantaneous rise in pH to above neutral when we reproduced their reduction conditions. These results, in addition to our finding that fibrils re-equilibrate very rapidly with the pH of the solution (Fig. 2), strongly suggest that the imminium compound dehydrohydroxymerodesmosine formed quickly and was reduced immediately with [1H]KBH₄ to hydroxylsylsinosonorleucine. This is supported by the fact that the synthesis of dehydrohydroxymerodesmosine is very rapid when lathrytic collagen fibrils are acted upon lysyl oxidase at pH 7.4 (Siegel and Lian, 1975; Siegel, 1976). The results of experiments in this report with insoluble collagen fibers indicate that dehydro-His-OHMerDes is a natural cross-link in collagen and is not an artifactual result.
of any type of reduction procedure. Therefore, it was of interest to determine the specific location of the His residue involved in the formation of dehydro-His-OH
dFrom soluble collagen that contained His-OH

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