**Relationship between the *Intra* and *Intermolecular* Cross-links of Collagen**

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**Abstract.** Spontaneous cross-linking in vitro, during incubation of purified collagen in nonstriated and native type fibrils, was studied to elucidate the relationship between *intra* and *intermolecular* cross-links. In nonbanded fibrils, the aldol condensation products of two allysyl residues, previously shown to constitute the *intramolecular* cross-link, formed spontaneously and could be isolated as such, whereas in native type fibrils, these compounds were found to be incorporated into an *intermolecular* cross-link; after chemical reduction they could not be isolated. Similar results were obtained from studies on a native collagenous tissue, rat tail tendon. It is suggested that the *intramolecular* cross-link is not a separate entity but only an intermediate of an *intermolecular* cross-link and that its existence in solubilized collagen is a result of the extraction procedure. The quantitative formation of *intramolecular* cross-links in vitro and identification of the cross-link compound are also reported.

The unusual mechanical stability of the collagen fibril is largely dependent upon the formation of covalent *intermolecular* cross-links. The partial and variable solubility of collagen in dilute acid suggests that some of these bonds may be weak and reversible, and the decreasing solubility with time may indicate a progressive spontaneous alteration in these bonds.

Both *intramolecular* and *intermolecular* cross-links have been found and some of the precursors and actual cross-link compounds have been isolated and identified.1–4 However, little data have been obtained bearing on the relationship between the two, and opinions expressed by different groups of investigators have not been in agreement.5,6

It has been shown that in rat- and chick-skin collagens, oxidative deamination of specific lysyl residues located near the NH₂-terminals of the adjacent alpha chains produces aldehydes (allysine), which react by aldol condensation to form the *intramolecular* cross-link.2,6 Such an aldol condensation product has been isolated from solubilized chick-7 and rat-skin8 collagens and identified.

Piez1(b) has suggested the possibility of formation of an *intermolecular* cross-link via the reaction of the aldehydic function of the aldol condensation product in one molecule with another reactive group in an adjacent molecule.

The first *intermolecular* cross-link isolated2 was from rat tail tendon collagen and was identified as hydroxylysinonorleucine after reduction with NaBH₄.3 They suggested that this compound was derived via Schiff base formation between...
an allysyl residue and the ε-amino group of a hydroxylysyl residue on another molecule. They also observed yet another intramolecular cross-link compound, derived in part from lysyl residues, eluting in the basic region of their amino acid chromatograph, but were unable to establish its structure. More recently, lysinonorleucine was recognized as an intermolecular cross-link in collagen.4 Its biosynthetic origin is presumably analogous to that of hydroxylysinoorleucine.

Kang et al.4 studied the relationship between the intra and intermolecular cross-links in fibrogenesis in vitro by the heat gelation system.10 They observed during fibril formation that the intramolecular cross-link (the aldol condensate) disappeared with concomitant formation of a basic compound eluting near histidine on chromatography, suggesting that the intramolecular cross-link participates in intermolecular cross-link formation. A more recent study indicates a precursor-product relationship between the aldol condensate and the "post-histidine" compound.11

This study extends the observation to tissues in vivo and suggests that the intramolecular cross-link is not a separate entity, but rather a part of an intermolecular cross-link, confirming Piez’s earlier suggestions.10

Materials and Methods. Preparation of collagen: Neutral salt-soluble collagen was extracted from the skins of 3-week-old white Leghorn chicks that had been fed a commercial diet containing 0.4% p-phenylamine for 2 weeks, and purified.12 Soluble collagen was prepared from the tail tendons of 100–150 g Sprague-Dawley rats by extracting with 0.5 M acetic acid, and purified identically to chick skin collagen. In some experiments, the tendons were used without prior solubilization but after thorough washing with cold 0.16 M NaCl, buffered with 0.05 M Tris, pH 7.0, which did not extract any detectable amounts of collagen as judged by hydroxyproline determinations.

Chromatography: The constituent subunit chains of collagen were fractionated on carboxymethyl (CM)-cellulose.4 Phosphocellulose chromatography of cyanogen bromide peptides was described.2 Molecular sieve chromatography was on columns of Bio-Gel beads in 0.1 M acetic acid.

In vitro fibril formation: The method was a modification4 of the heat gelation system.10 Aliquots of solutions of purified collagen dissolved in 0.05 M Tris buffer, pH 7.5, containing either 0.16 M or 1.0 M NaCl, were incubated at 37°C. Under these conditions, the 0.16 M NaCl solution of collagen precipitated as typical native-type fibrils, with 640 Å periodicity, whereas the 1.0 M NaCl solution precipitated as amorphous, nonstriated fibrils. At various time intervals aliquots were withdrawn and dialyzed against 0.5 M acetic acid to dissolve any soluble fibrils, and then against 0.06 M sodium acetate buffer, pH 4.8 in preparation for CM-cellulose chromatography. Other samples were reduced directly with [3H]NaBH₄.

Reduction with sodium borohydride: Collagen in solution, the in vitro induced fibrils, and rat tail tendon were reduced with [3H]NaBH₄.4

Cyanogen bromide cleavage: CNBr digestion of the various solubilized and reconstituted collagen samples and the tail tendons was by the methods of Bornstein and Piez.3

Amino acid analysis: Samples were hydrolyzed in constant-boiling HCl or 2 N NaOH at 105°C for 24 hr. Analyses were performed on an automatic instrument equipped with a split-stream device. Radioactivity was assayed using Bray’s solution in an automatic liquid scintillation counter.

Results. In vitro formation of the cross-link: Nonstriated fibrils: The structural identification and relationships of the CNBr peptides and cross-link regions are schematically presented in Fig. 1. Nonstriated fibrils formed in
FIG. 1. Diagrammatic representation of cross-linking. Specific lysyl residues (×) located near the NH₂-terminus of each chain (A), e.g., α1-CB1 or α2-CB1 are converted to aldehydes (●, allysine) (B). It is now thought from studies in vitro that two adjacent aldehydes condense spontaneously to produce the intramolecular aldol cross-links (C and D). In the native fibrils (E), in which the molecules all face in the same direction and overlap each other in a 1/4 staggered array, intermolecular cross-links may form between allysine (●) and lysine or hydroxylysine (×) forming the compounds lysinonorleucine or hydroxylysinoonorleucine, or between the aldol and another reactive group (●). The latter has not been identified but apparently is not lysine. In non-striated fibri’s (F), where there is only a random order or parallel molecules, allysine or aldol cannot undergo further reaction since appropriate reactive groups are not sterically apposed. This diagram is modified from Piez (Figs. 3 and 4) to indicate more fully our interpretation of the experiments reported here.

vitro fail to become insoluble upon incubation (Gross, unpublished) and do not form covalent intermolecular cross-links. Such fibrils are not comprised of regularly staggered molecules but rather represent parallel arrays randomly oriented with respect to each other.

In Fig. 2 are presented the CM-cellulose elution patterns of δ-penicillamine-treated chick-skin collagen incubated in nonbanded fibril form for various periods of time (2b and 2c) along with that of the original sample (2a) that had not been incubated. Peaks were identified by amino acid analysis and mobility on acrylamide gel electrophoresis. As has been shown, the salt-extracted collagen from δ-penicillamine-treated animals contains only small amounts of cross-linked components as a result of selective reversible binding of the agent to aldehyde residues. δ-Penicillamine is removed during purification. With the passage of time, however, the relative and absolute quantity of β components increases with little diminution in recovery of total protein, indicating gradual formation during the incubation of intramolecular cross-links. There is little loss of solubility of non-banded collagen over the 7-day period. In order to examine the nature of the newly formed β components, aliquots of the seven-day samples were digested with CNBr after salts were removed by dialysis is against 0.1 M acetic acid.
Fig. 2. CM-cellulose elution patterns of the original collagen sample (a), the samples incubated in non-banded fibril forms at 37°C for 3 days (b) and for 7 days (c), and the samples incubated in native type fibril form at 37°C for 3 days (d) and 7 days (e). Each chromatogram was obtained from identical amounts of material.

The elution pattern of the pertinent peptide region from phosphocellulose is illustrated in Fig. 3b, along with a similar chromatogram of the CNBr peptides derived from a control sample that had not been incubated (Fig. 3a). The peptide α2-CB1Altd was eluted unretarded from phosphocellulose but could be separated from nonpeptide, ultraviolet-absorbing, forepeak material (not shown in Fig. 3) by chromatography on Bio-Gel P-4. The amount of peptide present in the original peak was determined by amino acid analysis of the aliquots obtained from P-4 chromatography. The peptides α1-CB1Altd and α2-CB1 eluted together as one peak from phosphocellulose. The amounts of each peptide present in the peak were determined by molecular sieve chromatography on Bio-Gel P-2 after tryptic hydrolysis; α2-CB1 was cleaved at its lysyl residue into two fragments, T1 and T2.13 No trypsin-sensitive bonds are present in α1-CB1Altd.

Characterization of β12-CB1, obtained from the in vitro incubated sample, by amino acid analysis, by molecular sieve chromatography on Bio-Gel P-4, and by
chromatography on phosphocellulose gave identical results with the similar peptide obtained from the purified \( \beta_{12} \) components of chick-skin collagen.\(^6\) Furthermore, amino acid analysis and scintillation spectrometry of the alkaline hydrolyzate of the \([^{3}H]NaBH_4\)-reduced peptide demonstrated the presence of a stoichiometric amount of the aldol condensation product of two residues of allysine.

The peptide \( \beta_{11}-\text{CB1} \) did not separate from \( \alpha_1-\text{CB2} \) on phosphocellulose, but could be separated from the latter after trypsin treatment of the unseparated peptides and rechromatography on phosphocellulose. \( \beta_{11}-\text{CB1} \) does not contain trypsin-sensitive bonds but \( \alpha_1-\text{CB2} \) contains a residue of arginine and is cleaved by trypsin into two fragments, \( \alpha_1-\text{CB2}-\text{T1} \) and \( \text{T2} \), which elute at positions different from \( \beta_{11}-\text{CB1}.\)\(^{13}\)

It can be seen that the control sample contains negligible amounts of \( \beta_{11}-\text{CB1} \) or \( \beta_{12}-\text{CB1} \), but the 7-day sample contains appreciable amounts of the peptides, indicating their formation during the incubation. It is also noted that with the formation of the cross-linked peptides, \( \beta_{11}-\text{CB1} \) and \( \beta_{12}-\text{CB1} \), there is a concomitant decrease in the amounts of \( \alpha_1-\text{CB1}^{\text{Ald}} \) and \( \alpha_2-\text{CB1}^{\text{Ald}} \) (compare 3a and 3b).
This observation further supports the conclusion that the cross-linked peptides are derived from the latter peptides.\textsuperscript{2,13,15}

That the formation of the intramolecular cross-link can proceed spontaneously \textit{in vitro} from the existing precursors is further supported by results obtained from amino acid analysis and scintillation spectrometry of alkaline hydrolyzates of 7-day samples after \([^{3}H]\)NaBH\(_4\) reduction; there is a marked increase in the amount of the aldol condensate. This aldol was shown to be the intramolecular cross-link in chick-skin and rat-skin collagens.\textsuperscript{7,8}

Reconstituted striated fibrils (640 Å period): In contrast, the collagen samples from d-penicillamine-treated animals, incubated in native type fibril form became progressively insoluble in 0.5 M acetic acid.\textsuperscript{15} This is reflected in the decreased amounts of protein that could be solubilized and chromatographed on CM-cellulose (Fig. 2d, 2e). However, the subunit components obtained from CM-cellulose chromatography were indistinguishable, in every respect examined, from the "normal" components prepared from acid-extracted collagen from normal animals (not treated with penicillamine).

The progressive decrease in the amount of collagen soluble after incubation suggested that during incubation intermolecular cross-links were formed, and that the cross-link compound of the β component that appeared during incubation might be incorporated into intermolecular cross-links. It was suspected that the fraction of collagen dissolved in 0.5 M acetic acid and chromatographed on CM-cellulose became soluble because of instability of unreduced intermolecular cross-links derived in part from the aldol condensate. The 7-day native type fibrils were stabilized by reduction with \([^{3}H]\)NaBH\(_4\); reduced collagen was digested with CNBr and fractionated on phosphocellulose (Fig. 3c). A separate sample of collagen reduced in nonbanded fibril served as control. Apparently, the reduction of the aldehydic groups with NaBH\(_4\) does not alter the chromatographic behavior of the aldehyde-bearing peptides (α1-CB\(_{1}\)\textsuperscript{14d}, α2-CB\(_{1}\)\textsuperscript{14d}, β\(_{1}\)-CB1, and β\(_{2}\)-CB1). The elution pattern of the control sample was very similar to Fig. 3b and is not presented. It can be seen that β\(_{1}\)-CB1 or β\(_{2}\)-CB1 is present in negligible amounts in the reduced native type fibril digest. Amino acid analysis and scintillation spectrometry of an alkaline hydrolyzate of the reduced 7-day native fibrils showed that the aldol condensate, previously shown to be present in the intramolecular β component, was no longer detectable. However, a new radioactive compound eluting near histidine was present in addition to hydroxylysinoonorleucine and lysinoonorleucine. This "post-histidine" peak is derived in part from the aldol condensate, but its chemical structure has not been established.\textsuperscript{4,11}

Studies on intact rat-tail tendon: Since the data obtained from \textit{in vitro} fibrogenesis suggested that the intramolecular cross-link becomes incorporated into the intermolecular cross-link in native type fibrils, collagen in native tissue was examined. Rat-tail tendons from young animals were used since they are available in much purer form than dermal collagenous tissue, and are almost completely soluble in 0.5 M acetic acid. CM-cellulose chromatography of solubilized tendon collagen and characterization of its fractions by amino acid analysis and acrylamide disc electrophoresis showed that, as known previously,\textsuperscript{16}
over 50% of the protein consists of β-components. Analyses of the CNBr peptides obtained from the NH₂-terminal cross-link region of solubilized collagen revealed the presence of β₁₂-CB1 and β₁₁-CB1 in amounts expected from the contents of β₁₂ and β₁₁. Furthermore, the cross-links contained in those peptides were shown to be the aldol condensate of two residues of allysine as in the case of skin collagen.\(^7\)\(^8\) \(^9\)\(^{10}\)\(^{11}\) \(^{12}\)\(^{13}\)\(^{14}\) [\(^3\)H]NaBH\(_4\) reduction of the tendon rendered it insoluble to 0.5 M acetic acid. Phosphocellulose chromatography of a CNBr digest of the reduced tendon, and analyses of the fractions in a manner similar to that applied to chick-skin fibrils, revealed the absence of the peptides derived from the \textit{intramolecular} cross-link. Furthermore, amino acid analyses and scintillation spectrometry showed the presence of the \textquoteleft\textquoteleft post-histidine\textquoteright\textquoteright compound along with hydroxylsiononorleucine but the aldol condensate was no longer present in significant amounts. Thus, the results obtained from studies of the intact tendon were in every respect analogous to those reported in detail on the fibrogenesis system \textit{in vitro}.

**Discussion.** Evidence to date clearly indicates that the \textit{intramolecular} cross-link results from an aldol condensation of two lysine-derived aldehydes located on the adjacent α chains near the NH₂-termini.\(^2\)\(^6\) The enzyme(s) mediating the deamination of the ε-amino group of the lysyl residues has been detected in extracts of chick tibiae and partially purified by Pinnell and Martin.\(^7\) One of the aims of the present study was to determine whether the aldol condensation proceeds spontaneously once the reactive groups have been formed and to identify the product. Gross and Martin\(^8\) and Gross\(^9\) reported an increase in the content of β components after incubation of solubilized collagen in native type fibril form and similar observations were later reported by Deshmukh and Nimni\(^10\) in more detail. However, their low recovery of protein after incubation left some indeterminacies in interpreting the apparent increase in the β content, since only a fraction of collagen was solubilized and examined. Schiffman and Martin\(^11\) have presented evidence to show that \textit{intramolecular} cross-links formed \textit{in vitro} were derived from the same peptide region where \textit{intramolecular} cross-linking occurs \textit{in vivo}; however, the newly formed cross-link compound was not identified.

In the present study, unequivocal evidence was obtained to show that the new β components were formed during incubation \textit{in vitro}. In the experiment with reconstituted collagen in the non-banded form, recovery of protein after the incubation period was essentially complete; hence, no ambiguity should exist in concluding that the β components were formed at the expense of the α chains. Furthermore, the newly formed cross-link compound was identified and its location in the NH₂-terminal peptide region confirmed. \textit{Intramolecular} cross-linking occurs in nonbanded fibrils since the reactive aldehydic groups are closely apposed to each other within a given molecule.\(^2\)\(^6\)

A more revealing aspect of this study was the role of the aldol cross-link in the native type fibril. Tanzer\(^6\) noted that \textit{intermolecular} cross-link formation requires a precise alignment of the collagen molecule with exact apposition of the functional groups involved since the \textit{intermolecular} cross-links do not form in any fibril types other than the native fibril. Thus, in nonstriated fibrils, the aldol
cross-links form but the process stops there, presumably due to the lack of an apposing functional group. In native fibrils the aldol cross-link combines with a reactive group in a neighboring molecule to form an intermolecular cross-link because of the proper steric association. This reaction occurs in fibril formation in vitro as well as in vivo. These events are diagrammatically presented in Fig. 1.

No aldol condensate can be detected in intact rat-tail tendon fibrils, although it becomes detectable if the tendon is solubilized with acetic acid. In this sense, the intramolecular cross-link is a result of the extraction procedure and not a separate entity in the native tissue, except as an intermediate in intermolecular cross-link formation.

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