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Cross-Linking of Collagen

Endogenous aldehydes in collagen react in several ways to form a variety of unique covalent cross-links.

Marvin L. Tanzer

The connective tissue protein, collagen, is the most abundant protein in higher animals where it occurs primarily as extracellular, insoluble fibers. These fibers account for a large part of the organic mass of skin, tendon, blood vessels, bone, teeth, cornea, and vitreous humor. Collagen also provides the framework for most of the parenchymal organs, either in its fibrous form or organized in basement membranes. Because of the ubiquitous distribution and abundance of this structural protein it has been investigated by scientists working in a wide variety of disciplines and specialties ranging from such applied areas as suture production and leather manufacture to the more fundamental aspects of polymer chemistry and the structure-function studies of the protein chemist. In this article I discuss in some detail the structure and function of collagen. In particular I present arguments that (i) the insolubility of collagen fibers is primarily a conseqence of covalent cross-linking; (ii) the covalent cross-links arise from modified amino acids which contain carbonyl groups; and (iii) the molecular packing of collagen monomers into the polymer (fibril) probably specifies which cross-links are formed.

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Collagen Behavior

Although all proteins are unique in their structure and action, there are often similarities among proteins which

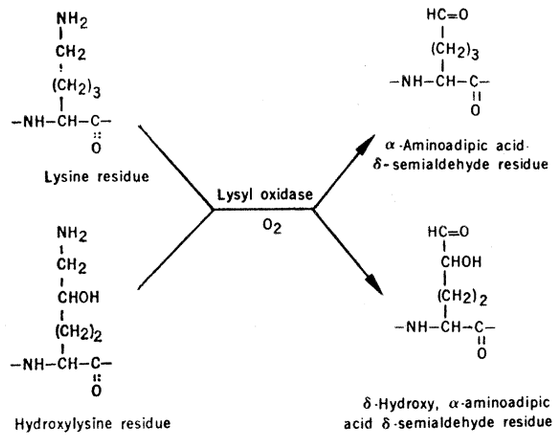


Fig. 1. Formation of collagen aldehydes by enzymatic action of lysyl oxidase on collagen molecules (*5*).

allow them to be grouped into classes. Originally, collagen was grouped with the "fibrous" proteins, keratin, myosin, and fibrinogen, but later it became apparent that only collagen and another connective tissue protein, elastin, can be considered related in any way. These two proteins share common features of cross-linking and, indeed, the studies of elastin cross-linking preceded those of collagen and provide an important reference point (*1*). Collagen can also be grouped with those proteins which undergo self-assembly into macromolecular structures, and is often cited as one of the original prototypes of self-assembly systems (*2*). However, no other similarities seem to exist between collagen and other proteins which polymerize into defined structures. Moreover, there are a number of features of molecular structure which characterize collagen as a highly unique and unusual protein. These include the content of three polypeptide chains (virtually all oligomeric proteins have an even number of chains), a specific helical conformation which is found in no other protein, and the presence of hydroxyproline and hydroxylysine, which are formed at the polypeptide level by specific hydroxylases. In addition, some of the hydroxylysine side chains contain substituted *O*-galactosyl and *O*-galactosyl-β-glycosyl residues. Finally, the high content of proline and glycine and the repetitive positioning of glycine at every third amino acid locus account for many of the molecular features of collagen, particularly its unique helical conformation.

The ability of collagen solutions to polymerize readily into collagen fibrils, which are indistinguishable from those found in vivo, has been extensively exploited as a model system of collagen fibrogenesis (*2*). This model system has an interesting property that resembles a phenomenon that occurs in vivo: the collagen fibrils show a progressive, increasing insolubility and eventually can only be dissolved by using strenuous denaturing conditions (*3*). Thus, not only is the capacity for self-assembly intrinsic to the collagen molecule, but so is the ability for the assembled polymer to exhibit progressive changes in its properties. This latter phenomenon can, in part, be attributed to the presence of reactive carbonyl moieties in collagen, which generate intermolecular cross-links, potentially uniting all of the collagen molecules into a continuous polymeric network.

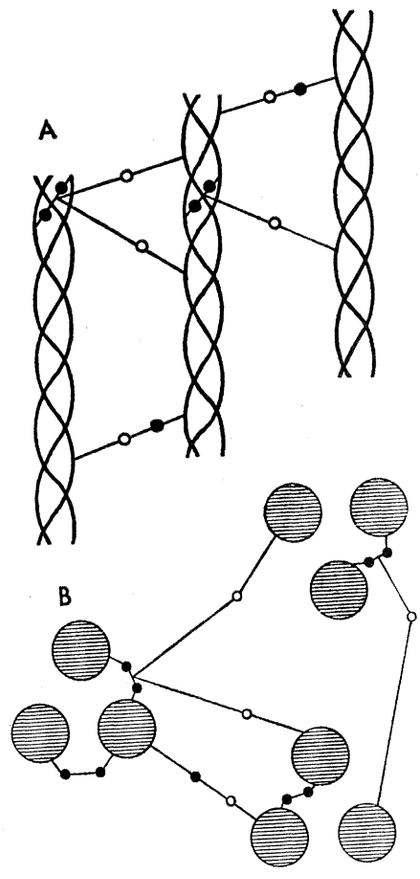


Fig. 2. General scheme of intermolecular cross-link formation, by reaction of collagen aldehydes with other collagen amino acids. (A) Lateral view. (B) Cross-sectional view. ●, Aldehydic groups; ○, other reactive groups.

Carbonyl Compounds

As is common in biochemistry, insight into a fundamental process was provided by the use of selective inhibitors. The lathyrogens comprise a group of nucleophilic compounds, exemplified by β-aminopropionitrile (BAPN), which specifically inhibit collagen cross-linking. The phenomenologic effects of BAPN administered to young developing animals include the loss of tissue tensile strength, spontaneous aortic rupture, abdominal herniations, and ultimately, severe bony deformations (*4*).

The collagenous architecture appears to be normal in the tissues but, in contrast to normal animals, most of the collagen fibers are readily dissolved by simply cooling the tissues. Thus, self-assembly of collagen molecules into collagen fibers is unaltered by the lathyrogens but the fibers do not become insoluble. Isolated lathyritic collagen also polymerizes normally in vitro, but the rate of fibril insolubility is markedly retarded compared with normal collagen fibrils. This abnormal behavior can now be explained by the fact that BAPN inhibits an enzyme (*5*) that converts certain collagen lysine and hydroxylysine ε-amino groups into aldehydes (Fig. 1). These aldehydes react with other amino acid side chains (Fig. 2) to form cross-links between adjacent collagen molecules. Because this cross-linking does not occur in collagen solutions, where the molecules are far apart, or in those collagen polymers in which the collagen molecules are incorrectly packed, specific requirements must be fulfilled for the development of successful cross-linking (*6*). Selective modification of the collagen aldehydes also prevents cross-linking, even if the molecules are properly packed in the fibril. Thus, the normal sequence of events appears to be: collagen molecules, which contain carbonyl groups, self-assemble into fibers which then become cross-linked because of reactions that occur between the carbonyl groups and other amino acids of adjoining molecules. Both rapid and slow reactions occur (*6, 7*), concomitant with the progressive, increasing insolubility.

Intermolecular Cross-Links

When aldehydes react with proteins, Schiff bases are commonly formed and usually such reactions involve the ε-amino group of lysine. Aldehydes intrinsic to proteins, such as the enzyme cofactor pyridoxal, as well as extrinsic aldehydes, such as formaldehyde, react in this fashion. Thus, one would anticipate that intrinsic collagen aldehydes might also form similar products, which could be converted to a more stable form by selective reduction with sodium borohydride. Furthermore, tritiated $NaBH_4$ labels the reduced compounds, allowing them to be detected and isolated. This experimental approach has been used with both reconstituted collagen fibrils and intact collagenous tissues (*6*, *8*); physicochemical studies show that exposure to $NaBH_4$ increases the collagen insolubility and that, once dissolved by denaturing solvents, the protein contains a complex mixture of cross-linked polypeptide chains of high molecular weight. These results can be unequivocally attributed to $NaBH_4$ reduction of the reducible cross-links which are generated from collagen aldehydes.

Comparison of the radioactive products obtained from hydrolyzates of NaB^3H_4-treated collagen solutions and reconstituted collagen fibrils show a conversion of carbonyl compounds into presumptive cross-links (Fig. 3). Similar chromatographic profiles are obtained from intact collagenous tissues, ranging from those which are almost entirely composed of collagen (tendon, demineralized bone, and dentine) to those such as skin which contain many other connective tissue components (*9*). Isolation and structural characterization of most of the radioactive amino acids has been recently accomplished and provides a new chapter in collagen biochemistry.

The two most abundant aldehydes in collagen are α-aminoadipic acid δ-semialdehyde and its hydroxylated analog (Fig. 1). These amino acids are formed by the action of lysyl oxidase (*10*) on collagen; they are most often found in abundance near the amino terminal of the polypeptide chains but are also found elsewhere in the chains (*11*), particularly in "older" collagens. Within the collagen molecule, two of the aldehydes condense to form an α,β-unsaturated aldol condensation product (Table 1) which is an intramolecular cross-link (*12*). This compound is quite stable, even occurring in small peptides obtained from the protein, and appears to occur only near the amino terminal of the collagen chains. It is destroyed by the conditions of acid hydrolysis but is relatively stable to base hydrolysis. (Consequently, it is not seen in the chromatograms shown in Fig. 3.)

Kinetic studies in which reconstituted collagen fibrils are used show that the aldol condensation product slowly disappears during sustained incubation of the fibrils, and a new substance is formed at a rate equivalent to the loss of the aldol condensation product (*7*). This new substance, the cross-link histidino-hydroxymerodesmosine (Table 1), is composed of equivalent amounts of the aldol condensation product, histidine, and hydroxylysine (*13*). Thus, potentially it can unite four polypeptide chains, joining two or more collagen molecules, depending upon which particular polypeptide chains contribute to its formation. The participation of histidine in this structure is especially significant because histidine accounts for less than 1 percent of the

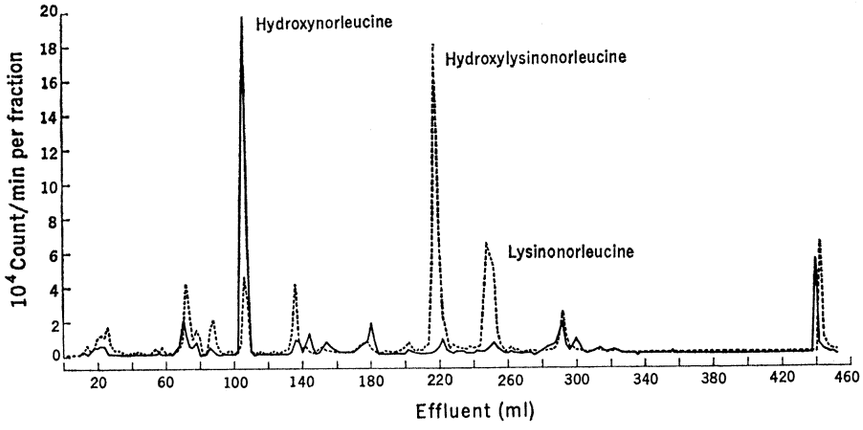


Fig. 3. Genesis of intermolecular cross-links from collagen aldehydes on reconstitution of native-type collagen fibrils from collagen molecules in solution. Collagen was reacted with NaB^3H_4, subjected to acid hydrolysis, and the hydrolyzate chromatographed upon ion-exchange resin columns (*6*) (———, collagen molecules; - - - -, reconstituted collagen fibrils).

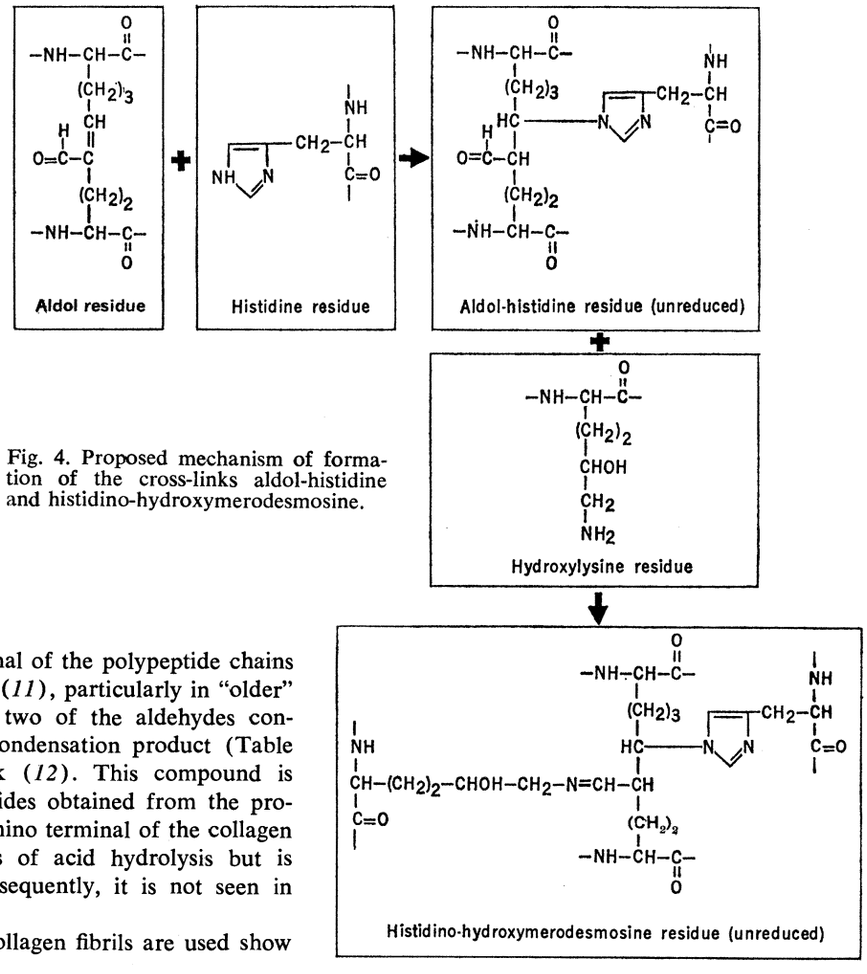


Fig. 4. Proposed mechanism of formation of the cross-links aldol-histidine and histidino-hydroxymerodesmosine.

amino acids in collagen and is located in very specific regions of the protein. Thus, precise molecular alignments must occur in the fibrils in order to bring together three rare components (Fig. 4) (hydroxylysine is present in most collagens in an amount equal to 1 percent of the amino acids). Furthermore, an analog of histidino-hydroxymerodesmosine, also containing histidine but lacking the hydroxylysine moiety, is abundant in cow skin collagen. This cross-link, aldol-histidine (Fig. 4), can potentially unite three polypeptide chains, most probably between adjacent collagen molecules since it is not present until the collagen is reconstituted into fibrils. Both aldol-histidine and histidino-hydroxymerodesmosine are also abundant in collagenous tissues, although the latter compound is common to all species studied whereas the former one is predominant only in cow skin collagen. Both compounds are functionally analogous to the tri- and tetrafunctional cross-links, merodesmosine and desmosine, found in elastin (*1*), but are structurally different.

Considerable similarity between the cross-links of elastin and collagen exists in the case of hydroxymerodesmosine, hydroxylysinonorleucine, dihydroxylysinonorleucine, and lysinonorleucine (Table 1). The trifunctional cross-link, merodesmosine (*1*) is present in elastin and small amounts of its monohydroxylated analog are detected in collagen (*13*). The difunctional cross-link, lysinonorleucine, is present in both proteins but occurs in relatively small amounts in collagen and may be formed simply as a consequence of incomplete hydroxylation of lysine (*14*).

This seems particularly true because hydroxylysinonorleucine is a major cross-link in many collagens and periodate degradation studies indicate that it arises by way of the Schiff base formed between hydroxylysine and α-aminoadipic acid δ-semialdehyde (*14*). Thus the known incomplete hydroxylation of lysine at specific loci would readily give rise to the analogous, nonhydroxylated Schiff base product. The difunctional cross-links and hydroxymerodesmosine also appear to be primarily intermolecular in nature because, unlike the aldol condensation product, they are not present in collagen molecules and are formed only when collagen is reconstituted into fibrils. Determination of the exact location of these cross-links would mean, of course, that their intermolecular nature would be specified.

The parallel studies of cross-linking in collagenous tissues and in reconstituted fibrils has recently led to the characterization of compounds which occur in the tissues but not in the reconstituted fibrils. These substances, N^{ϵ}-hexosylhydroxylysine (*15*), N^{ϵ}-glucosyllysine, and N^{ϵ}-mannosyllysine (*15*) are abundant in many collagenous tissues, especially skin, but their origin is uncertain. N^{ϵ}-Hexosylhydroxylysine must partially arise from collagen because of its hydroxylysine component, but the lysine-derived substances may well originate from other connective tissue components. The possibility of unusual artifacts also complicates the picture, particularly when intact tissues are investigated. For example, we have recently isolated small amounts of tritium-labeled N^{ϵ}-monomethyllysine from NaB^3H_4-reduced cow skin (*16*); this compound was probably formed as a result of interactions between formaldehyde and peptidyl lysines. (The formaldehyde may be in trace amounts in the solvents used to extract the collagenous tissues, or in those used during the $NaBH_4$ reduction.) Thus, the significance of the hexose-containing compounds is questionable at this time; isolation of larger units, such as peptides, which contain them, may provide a firmer basis for their being assigned a biochemical function.

Cross-Link Location

As the structures of the reducible, carbonyl-derived cross-links are elucidated the next logical step is to determine their location in the collagen polymer. Not only would such studies establish conclusively the intermolecular origin of the cross-links, but they could also provide insight into the specific packing arrangement of collagen molecules in the fibril. The latter is attainable because the entire sequence of amino acids in the polypeptide chains of collagen will soon be known and the linear arrangement of the cyanogen bromide peptides obtained from the collagen chains is already known (*17*). Thus, peptides containing covalent cross-links which unite two or more different regions of collagen molecules can potentially be related to the appropriate portions of the molecule.

Sufficient information is already available to indicate that hydroxylysinonorleucine, dihydroxylysinonorleucine, lysinonorleucine, aldol-histidine, and histidino-hydroxymerodesmosine are contained in peptides which arise from natural or reconstituted collagen fibrils (*13, 18–21*). Furthermore, isolation of peptides from CNBr digests of rat tail tendon collagen have shown that hydroxylysinonorleucine is present in a peptide which contains two different regions of the collagen molecule (*18*) and that another peptide containing two different regions of collagen plus an unidentified cross-link, can be isolated from cartilage collagen (*19*). Smaller peptides, containing hydroxylysinonorleucine (*20*) and dihydroxylysinonorleucine (*21*), have been isolated from reconstituted calf skin collagen fibrils and from bovine dentine collagen, respectively. The two histidine-containing cross-links are present in large CNBr-derived peptides which have been purified from calf skin collagen (*13*). As these studies progress, it may eventually be possible to construct a map illustrating the packing arrangement of collagen molecules and the location of specific intermolecular cross-links.

Cross-Link Fate

Since the carbonyl-derived cross-links are readily reducible by exogenous reagents, it is reasonable to suspect that they may also become naturally reduced. This appears to be the case in both natural and reconstituted collagen fibrils (*9, 22, 23*). In the former instance, isotope dilution studies in which $NaBD_4$ was used in conjunction with mass spectrometry showed that 25 to 50 percent of hydroxylysinonorleucine and dihydroxylysinonorleucine became reduced in vivo. In studies of reconstituted fibrils, in which the collagen had been biosynthetically labeled with [^{14}C]lysine, four nonreducible cross-links developed progressively and one of these cross-links incorporated protons from the aqueous solvent (*23*). Two of the four cross-links were identified as lysinonorleucine and hydroxylysinonorleucine by chromatography. Thus, two independent studies of natural and reconstituted fibrils showed that some mechanism exists for reduction of Schiff base cross-links.

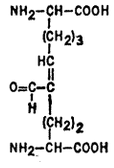
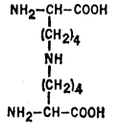
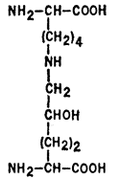
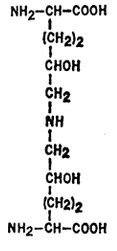
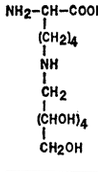
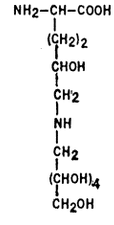
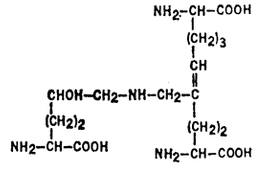
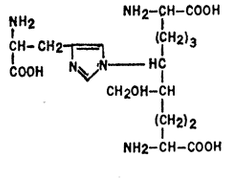
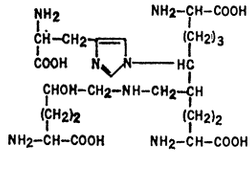
It is conceivable that atoms, other than protons, may react with the Schiff base double bond, perhaps accounting for some of the other nonreducible

products detected in reconstituted fibrils. The proposed origin of aldol-histidine (Fig. 4) is a prototype reaction, indicating that Michael addition of the imidazole nitrogen to an "activated" double bond occurs. Moreover, since dehydrohydroxylysinonorleucine and dehydrodihydroxylysinonorleucine can be considered in equilibrium with their keto-amino forms (*24*), other reactions are potentially possible, involving the ketone moiety. Exogenous additions to the Schiff base form of the cross-links are readily accomplished by cyanide and ammonia in a modified Strecker reaction (*25*), and provide an independent confirmation of the existence and structure of intermolecular cross-links.

The problem of determining quantitatively the molar amounts of carbonyl compounds and carbonyl-derived cross-links in collagen has not received as much attention as the qualitative studies of cross-link structure. The emphasis on structural determination has, of course, been generated by our greater curiosity in learning new structures than in measuring their abundance. Another factor is that the technical problems of maintaining stability under conditions of hydrolysis and the conversion of the cross-links to nondetectable products will allow only minimal quantitative estimates to be made. It has been shown that the amount of α-aminoadipic acid δ-semialdehyde per collagen chain ranges from 0.5 to 3 moles, depending upon the source and "maturity" of the collagen (*11*, *23*). In reconstituted fibrils, virtually all of this aldehyde generates cross-links (*23*), indicating a significant, quantitative role of the compound in collagen cross-linking. This result, taken in conjunction with the dramatic effects of the lathyrogens, conclusively document the importance of carbonyl compounds in collagen cross-linking.

The relationship of the carbonyl-derived cross-links to biologic phenomena such as embryonic development, aging, disease, and deficiency disorders has been investigated. The compounds are present in all collagenous tissues, both vertebrate and invertebrate, and their relative abundance in such tissues varies greatly (*26*). They are also found in several basement membrane collagens (*27*). Since the origin of a given cross-link is dependent upon both the nature of the carbonyl precursor and its specific reaction with another amino acid in correct proximity, at least two variables can affect the relative abundance of a

Table 1. Collagen cross-links.

Cross-link	Origin	Comments
	α,β-Unsaturated "aldol"	
	Aldol condensation product of two α-aminoadipic acid δ-semialdehydes (*12*)	Found only near NH_2-terminal of collagen chains, acting as an intramolecular cross-link; present in elastin
	Lysinonorleucine	
	Reduced Schiff base product of lysine and α-aminoadipic acid δ-semialdehyde (*14*)	Common to both collagen and elastin
	Hydroxylysinonorleucine	
	Reduced Schiff base product of either lysine and δ-hydroxy, α-aminoadipic acid δ-semialdehyde, or hydroxylysine and α-aminoadipic acid δ-semialdehyde (*8*)	Abundant in most collagens
	Dihydroxylysinonorleucine	
	Reduced Schiff base product of hydroxylysine and δ-hydroxy, α-aminoadipic acid δ-semialdehyde (*9*)	Most abundant in mineralized collagens
	N^{ϵ}-glucitol-lysine, N^{ϵ}-mannitol-lysine	
	Reduced Schiff base product of lysine and either glucose of mannose (*15*)	Origin unknown; present in "older" connective tissues
	N^{ϵ}-hexosylhydroxylysine	
	Reduced Schiff base product of hydroxylysine and a hexose (*15*)	Origin partially from collagen; present in connective tissues which contain polysaccharides
	Hydroxymerodesmosine	
	Reduced Schiff base product of unsaturated "aldol" and hydroxylysine (*13*)	Analogous to merodesmosine of elastin
	Aldol-histidine	
	Michael addition product of unsaturated "aldol" and histidine, isolated after reduction (*13*)	Abundant only in cow skin collagen
	Histidino-hydroxymerodesmosine	
	Reduced Schiff base product of aldol-histidine and hydroxylysine (*13*)	Abundant in most collagens, isolated as two isomeric forms

cross-link. (Of coure, many other factors may also affect the development of specific cross-links.) Thus, the determination of the relative abundance of the reducible cross-links, while potentially interesting, may not provide direct insight into various biologic phenomena which involve collagen. For example, although the abundance of various carbonyl compounds changes as a function of wound healing, age, vitamin D deficiency, animal species, source of tissue, and a hereditary disorder of connective tissue (*28*), it is not clear what factors are responsible for these differences. While it is tempting to speculate that a "Rosetta stone of aging" may originate in the covalent cross-links of collagen, it is too early to come to any reasonable conclusions on this subject. However, it does seem probable that once more structural information is available, the mechanisms of the regulatory processes can be probed in detail.

Summary

The formation of collagen cross-links is attributable to the presence of two aldehyde-containing amino acids which react with other amino acids in collagen to generate difunctional, trifunctional, and tetrafunctional cross-links. A necessary prerequisite for the development of these cross-links is that the collagen molecules be assembled in the naturally occurring fibrous polymer. Once this condition is met, cross-linking occurs in a spontaneous, progressive fashion. The chemical structures of the cross-links dictate that very precise intermolecular alignments must occur in the collagen polymer. This seems to be a function of each specific collagen because the relative abundance of the different cross-links varies markedly, depending upon the tissue of origin of the collagen.

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Evaluation of Instruction

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An essential role of teaching institutions is to teach—that is, to transmit knowledge, skills, and attitudes (*1*). The degree of success different institutions achieve in this respect may be viewed as dependent on the intellectual caliber of both its incoming students and its faculty and on the teaching effectiveness of that faculty. To ensure high intellectual caliber among their incoming students, many institutions place heavy emphasis in their admission policies on the applicant's score on national normative examinations such as the Scholastic Aptitude Test, the Graduate Record Examination, the Law School Admission Test, the Medical College Admission Test, and the Dental Aptitude Test. Likewise, to ensure teaching effectiveness among their faculty, teaching institutions may be reasonably expected to foster it by their promotion policies. Such institutional efforts, however, are likely to be hindered by the lack of agreement regarding the criteria upon which to base methods for measuring teaching effectiveness quantitatively.

The high correlation found between academic rank and publication output (*2*) supports the widespread impression that publication output has been the major determinant in promotion decisions (*3*). As the premise that good researchers are ipso facto good teachers is being challenged (*2*, *4*, *5*), increasing attention has been given to more direct methods of measuring teaching effectiveness (*2*, *6–9*). Among those proposed are (i) student ratings of instruction and (ii) class performance in examinations. Although both methods have certain disadvantages, the expressed desire of students to participate in the evaluation of courses and the view that students, as customers of the educational service, are in the best position to evaluate its worth (*7–9*) have resulted in increasing use of student ratings (*2*). This can be considered a reasonable devel-

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