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Stable, Nonreducible Cross-Links of Mature Collagen†

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ABSTRACT: During in vivo maturation, and also during in vitro incubation with physiological buffers, native collagen fibers display a progressive increase in tensile strength and insolubility. Paralleling these physiologically important changes is a progressive loss of the reducible cross-links which initially join the triple-chained subunits of collagen fibers. Although there is evidence suggesting that the reducible cross-links are gradually transformed into more stable, nonreducible cross-links during maturation, the nature of the transformation process and the structure of the stable "mature" cross-links has remained a mystery. In order to test the possibility that cross-link transformation involves addition of a nucleophilic amino acid residue to the reducible cross-links, histidine, arginine, glutamate, aspartate, lysine, and hydroxylysine residues were chemically modified, and the effect of each modification procedure on the in vitro transformation of reducible cross-links was ascertained. The results of these experiments indicated that destruction of histidine, arginine, glutamate, and aspartate residues has no measurable effect on the rate and extent of reducible cross-link transformation in hard tissue collagens. In contrast, modification of lysine and hydroxylysine residues with a wide variety of specific reagents completely blocks the transformation of reducible cross-links. Removal of the reversible blocking groups from lysine and hydroxylysine residues then allows the transformation to proceed normally. These results indicate that collagen maturation involves nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible cross-links, yielding derivatives which are not only more stable but also capable of cross-linking more collagen molecules than their reducible precursors.

Collagen, the most abundant protein of higher vertebrates, is unusual in several respects. Not only do its large, triple-chained subunits undergo spontaneous self-assembly to form fibrils, but in addition, these fibrils exhibit a unique maturation process which gradually transforms them into more stable aggregates (Fowler and Bailey, 1972). Furthermore, this process appears to occur more quickly in vitro and in the collagen fibers of healing wounds than in normal, developing tissues. Collagen, which is formed extracellularly by proteolytic cleavage of a soluble precursor, procollagen (Bellamy and Bornstein, 1971; Layman et al., 1971), contains reactive aldehyde residues which are produced extracellularly by oxidative deamination of a few lysine and/or hydroxylysine residues (Piez, 1968). When the collagen molecules aggregate to form native fibrils, aldehyde residues on one molecule can then react with lysine or hydroxylysine residues on another molecule to form aldimine cross-links (Bailey and Peach, 1968; Tanzer et al., 1970; Mecha-

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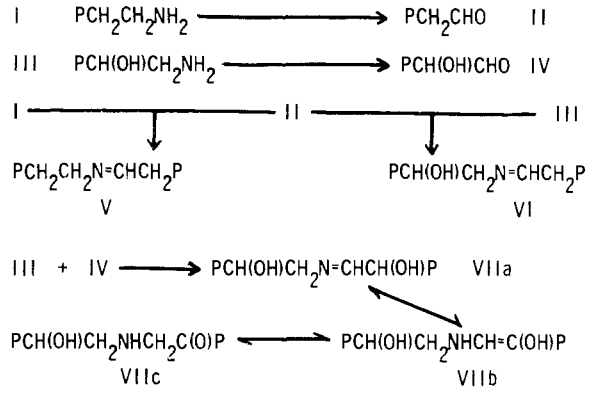

FIGURE 1: Formation of reducible collagen cross-links.

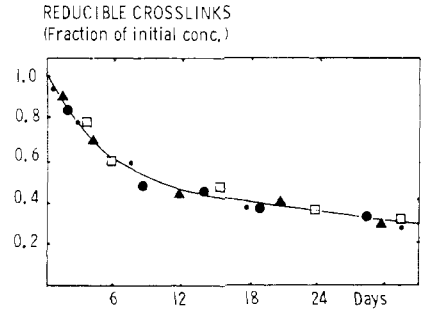

FIGURE 2: Reducible cross-link transformation rates. (—) Collagen modified at histidine (▲), at aspartate and glutamate (□), at arginine (●), and unmodified (•).

nic et al., 1970; Davis and Bailey, 1971). The structures of the aldehydes (II and IV) and the aldimine cross-links (V, VI and VII) are indicated in Figure 1. It has recently been demonstrated that cross-link VII can isomerize to the more stable α-ketoamine, VIIc, via the enaminol, VIIb (Robins et al., 1973; Eyre and Glimcher, 1973; Davis, 1973a). However, little is known about the role of these reducible cross-links in stabilizing mature collagen fibers, beyond the fact that they disappear during maturation (Robins et al., 1973).

It was suggested several years ago (Bailey, 1968) that the reducible cross-links are intermediates which are transformed into more stable, nonreducible cross-links. Furthermore, it was proposed in a recent paper (Davis, 1973a) that the nonreducible cross-links of mature collagen could be formed by addition of nucleophilic amino acid residues of collagen to the electrophilic double bonds of the reducible cross-links. The purpose of the present investigation was to test this proposal and to deduce the probable structure of the mature cross-links.

Experimental Section

Materials

Calf bone collagen and dentin collagen were prepared by the following procedure. First, pieces of crushed bone or dentin were repeatedly extracted at 20° with 0.5 *M* EDTA (pH 7.4) until only trace amounts of calcium remained. The demineralized bone or dentin was then extracted for three 24-hr periods with 1 *M* NaCl (pH 7.4, 0.05 *M* Tris). This removes phosphoproteins and other soluble products (Butler et al., 1971). Insoluble bone and dentin collagens thereby obtained were cut into pieces (about 1 mm^3 in size) and stored at 3° in 0.15 *M* NaCl (pH 7.4, 0.05 *M* Tris) under a layer of toluene until required. At this temperature, negligible transformation of the reducible cross-links occurs.

[^{14}C]Glycine ethyl ester was synthesized by heating 0.79 mg of [^{14}C]glycine (50 mCi ^{14}C) in 10 ml of HCl-saturated ethanol at 75° for 4 hr in a sealed tube. Nonradioactive glycine ethyl ester hydrochloride (789 mg) was then added to dilute the radioactive ester. Crystals obtained by storing the ester overnight at −20° were recrystallized to constant specific activity before use in free carboxyl group assays.

The trimer of 2,3-butanedione was synthesized as described by Grossburg and Pressman (1968). All other chemicals were commercially available products.

Methods

Rate of Reducible Cross-Link Transformation. The rate and extent of reducible cross-link transformation in modified collagens, unmodified collagens, and controls was determined by incubating appropriate samples at 37° in 0.15 *M* NaCl (pH 7.4, 0.05 *M* Tris or phosphate buffer); 20–30 mg was removed at intervals for reduction with $NaBH_4$, and the reducible cross-links were determined as previously described (Davis, 1973b).

Modification of Acidic Residues. The glutamate and aspartate residues of collagen were blocked by condensation with glycine ethyl ester or NH_3 in the presence of *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide, as described previously (Davis and Walker, 1972). The number of free carboxyl groups remaining after modification was determined by treatment with [^{14}C]glycine ethyl ester and carbodiimide in 6 *M* guanidine (Hoare and Koshland, 1967).

Acetylation of Lysine and Hydroxylysine. Collagen was equilibrated in 0.5 *M* borate buffer (pH 8.5) and blotted as dry as possible with filter paper. Then 600 mg of this collagen was stirred vigorously in 30 ml of the ice-cold borate buffer on an ice bath and treated with 1 ml of acetic anhydride. The pH was held at 8.5 ± 0.1 for 1 hr by addition of 5 *M* NaOH. Four additional treatments with acetic anhydride were performed as described above and the acetylated collagen was filtered off and washed repeatedly with H_2O and then 0.15 *M* NaCl (pH 7.4, 0.05 *M* Tris).

Ethoxyformylation of Basic Residues. Collagen (600 mg, blotted dry) was stirred overnight in 10 ml of 5 m*M* acetate buffer (pH 7.0) containing 1 mmol of ethoxyformic anhydride. After removal of the supernatant, the collagen was washed well with H_2O. Labile ethoxyformyl groups were then removed by treatment with 1 *M* NH_4OH at pH 7 as previously described for other proteins (Melchior and Fahrney, 1970).

Citraconylation of Lysine and Hydroxylysine. Samples of collagen (600 mg, blotted dry) were equilibrated at pH 8.5 in 7 ml of H_2O and stirred vigorously in a water bath at 20° while 0.7 ml of citraconic anhydride were added all at once. Addition of 5 *M* NaOH maintained the pH of the rapidly stirred, three-phase mixture at 8.5 ± 0.5 for the 10–15 min required for complete reaction or hydrolysis of the anhydride. Treatment with 0.7 ml of citraconic anhydride at pH 8.5 ± 0.5 was repeated once, twice, or three times if more extensive modification was required.

Complete removal of citraconyl groups from lysine from hydroxylysine residues was effected by treatment with 0.1 *M* HCOOH (pH 3.5) at 37° (Dixon and Perham, 1968).

Assay for Unmodified Lysine and Hydroxylysine Residues. The content of free lysine and hydroxylysine residues in acetylated, citraconylated, ethoxyformylated, maleated, and unmodified collagens was determined by stirring 30-mg

samples in 1 ml of 1.04 *M* acrylonitrile in 0.075 *M* borate buffer (pH 10.5) for 4 days at 20°. This completely and irreversibly cyanoethylates all unmodified lysine and hydroxylysine residues in collagen, as shown by amino acid analysis of the acid hydrolysates.

Further Modification of Citraconylated Collagen. Collagens which had been partially citraconylated (until transformation was blocked) were completely modified with ethoxyformic anhydride, acetic anhydride, or acrylonitrile as described above for unmodified collagens. The lysine and hydroxylysine residues which had been reversibly blocked with the citraconyl group were then completely regenerated by treatment with 0.1 *M* HCOOH as before. All these collagens were assayed for cross-link transformation and for content of unblocked lysine and hydroxylysine, as described above.

Carboxymethylation of Histidine. Collagen samples (400 mg) which had been extensively citraconylated to protect lysine and hydroxylysine residues were treated with 10 ml of 2 *M* iodoacetic acid at pH 7 in phosphate buffer until amino acid analysis indicated that carboxymethylation of histidine was complete. After extensive washing and removal of the citraconyl groups to regenerate lysine and hydroxylysine, the carboxymethylated collagen was assayed for cross-link transformation in 0.15 *M* NaCl, as described above.

Cyclohexanedione Modification of Arginine. Arginine was modified by stirring 600 mg of citraconylated collagen with 560 mg of 1,2-cyclohexanedione in 0.1 *M* triethylamine at pH 11 for 1–6 days in the dark (Liu et al., 1968). When all the arginine was blocked (as determined by amino acid analysis), the citraconyl groups were removed and the modified collagens assayed for cross-link transformation.

Butanedione and Glyoxal Modification of Arginine. Citraconylated collagens were modified with the trimer of butanedione as described by Grossburg and Pressman (1968) and also with glyoxal, as described by Nakuya et al. (1967). After removal of the citraconyl protecting group, the modified collagens were assayed for cross-link transformation.

Thermal Regeneration of Reduced Cross-Links. Bone, dentin, tendon, and cartilage collagens were incubated in 0.15 *M* NaCl at 37° until less than 0.06 residue/1000 of VII and VIIc was present. Samples of 150 mg were then heated at 70° in 0.5 ml of either 0.1 *M* HCl or 0.15 *M* NaCl (pH 7.4) for 10–60 min in a 1-ml test tube. After cooling, enough 5 *M* KOH was added to raise the pH to 10 or 11, and the mixture reduced for 1 hr with 10 mg of $NaBH_4$. The mixture was then acidified, evaporated to dryness, hydrolyzed in constant boiling HCl at 110° for 22 hr, and analyzed for cross-links as described previously (Davis, 1973a).

Cross-Link Transformation at Various Temperatures and pH's. The rate and extent of reducible cross-link transformation at pH 5, 6, 7, and 8.5 were studied in phosphate buffer at 37°, as described above for pH 7.4 buffer. Cross-link transformation at 3 and 23° was also studied in pH 7.4 buffer over several months in order to ascertain the temperature dependence of the process.

pH Controls. Bone and dentin samples were incubated at pH 11 (0.1 *M* triethylamine) for 2 weeks at 23°, or in pH 3.5 formate for 1 week at 23°, and then assayed for cross-link transformation in neutral saline at 37°.

Results

The results from specific modification experiments indicate that glutamate, aspartate, histidine, and arginine residues have no role in the transformation of reducible cross-links. Indeed, modification of 96% of the arginine, 80% of the histidine, and 98% of the acidic residues has no discernible effect on either the rate or extent of reducible cross-link loss during incubation in 0.15 *M* NaCl at 37° (Figure 2).

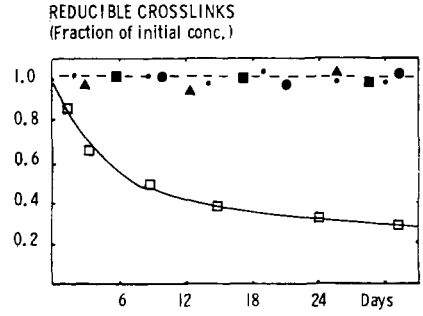

FIGURE 3: Reducible cross-link transformation rates. (- - -) Collagens modified with acrylonitrile (●), acetic anhydride (•), ethoxyformic anhydride (▲), and citraconic anhydride (□) to block lysine and hydroxylysine residues. (□) Collagens modified with citraconic anhydride, tested for blockage of transformation, and then demodified at pH 3.5

In contrast, modification of lysine and hydroxylysine residues completely blocks the cross-link transformation (Figure 3). Acetylation, which blocks 100% of the amino groups, ethoxyformylation, which blocks 92% of the amino groups, or citraconylation, which blocks from 30 to 50% of the amino groups, completely blocks the cross-link transformation. However, removal of the reversible citraconyl (or maleal) blocking groups from modified samples which fail to transform yields collagens which lose the reducible cross-links at rates indistinguishable from those observed with unmodified collagens. Modification of as few as eight lysine and three hydroxylysine residues with citraconic anhydride completely blocks the transformation of reducible cross-links.

Further modification of citraconylated collagens with acetic anhydride or with ethoxyformic anhydride blocks all unprotected amino groups, as indicated by acrylonitrile assays. Hence, removal of the citraconyl groups from citraconylated–acetylated, citraconylated–ethoxyformylated, and citraconylated–cyanoethylated collagens yields derivatives with 22 out of 33 lysine/hydroxylysine residues blocked. However, the 11 lysine/hydroxylysine residues unblocked by decitraconylation appear to be the only ones involved in cross-link transformation, since decitraconylation permits cross-link transformation despite the presence of large numbers of irreversibly cyanoethylated or acylated lysine and hydroxylysine residues.

Thermal denaturation experiments on mature bovine bone, dentin, cartilage, and tendon collagens indicate that the transformation process can be reversed by acid-catalyzed breakdown of the nonreducible cross-links at 70°. Indeed, after thermal treatment for 30 min in 0.1 *M* HCl, the content of reducible cross-links VII and VIIc rises from 0.04, 0.06, 0.02, and 0.01 residues per 1000 in mature bone, dentin, cartilage, and tendon, respectively, to 0.18, 0.13, 0.10, and 0.06, respectively. Thermal treatment at neutral pH is far less effective at regenerating VII and VIIc in bone and dentin collagens.

The rate of reducible cross-link transformation in unmodified collagen fibers was found to be very slow at 23°, and negligible at 3° (see Discussion).

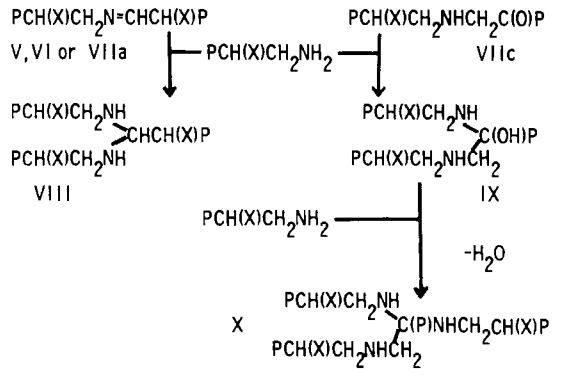

FIGURE 4: Formation of mature collagen cross-links. X = H or OH.

Discussion

It is evident from numerous glutamate, aspartate, histidine, and arginine modification experiments that these residues are not involved in the transformation of reducible collagen cross-links into the more stable, nonreducible cross-links of mature fibers. Aside from these residues, the other nucleophilic residues in collagen are glutamine, asparagine, serine, threonine, lysine, and hydroxylysine. Since modification of the ϵ-amino group of the latter two amino acid residues by a variety of techniques *completely* halts transformation of the reducible cross-links in vitro, it is extremely unlikely that other amino residues are essential for the transformation. Indeed, if some of the reducible cross-links were transformed by nucleophilic attack of residues other than lysine or hydroxylysine, one would expect lysine and hydroxylysine modification to partly halt cross-link transformation, but not to block it completely. However, the above observations do not eliminate the possibility that other nucleophilic amino acid residues could play a role in further transformation of cross-links derived from nonreducible transformation products of general structure IX (see Figure 4 and subsequent discussion). Nevertheless, our current working model is that cross-links of general structure IX are transformed only by nucleophilic attack of lysine or hydroxylysine residues (Figure 4), since this is the simplest hypothesis.

A role for lysine and/or hydroxylysine residues in the transformation of reducible cross-links is strongly suggested by a number of modification experiments. Acetylation, ethoxyformylation, citraconylation, and modification with maleic anhydride all totally inhibit the transformation process. Since removal of reversible blocking groups yields collagens which again undergo the cross-link transformation, blockage of the amino groups per se (and not merely structural alteration resulting from nonphysiological conditions during modification) must be responsible for inhibiting transformation. Control experiments in which collagen fibers were exposed to the acidic or alkaline conditions involved in several of these modifications for long periods indicate that pH's between 3 and 11 do not affect cross-link transformation.

Modification of as few as 11 lysine/hydroxylysine residues with citraconic anhydride completely inhibits transformation. Furthermore, acetylation or ethoxyformylation of such citraconylated collagens, followed by decitraconylation, yields products with the remaining 21 lysine/hydroxylysine residues blocked. These products undergo a transformation of reducible cross-links which proceeds at the same rate as in unmodified collagens, despite the presence of bulky blocking groups on 21 of the 33 lysine/hydroxylysine residues. It therefore seems very unlikely that the inhibition of cross-link transformation resulting from modification of 11 lysine/hydroxylysine residues can be attributed to steric perturbation of the collagen fiber by blocking groups. Indeed, if cross-link transformation were sensitive to steric perturbation, one might expect that the large glycine ethyl ester, carboxymethyl and cyclohexanedione groups introduced onto acidic residues, histidine residues and arginine residues, respectively, would affect transformation. However, in spite of the close proximity of three acidic residues, three histidine residues and three arginine residues to the known position of reducible cross-links on α_1 chains, no inhibition of cross-link transformation is observed. Hence, modification of lysine and hydroxylysine residues appears to block transformation of reducible cross-links by destroying their ability to initiate nucleophilic attack on the electrophilic double bonds of the reducible cross-links, and not by disrupting the structure of the fiber.

The 11 or so lysine and hydroxylysine residues modified by limited citraconic anhydride treatment are likely to be the most exposed ones, since this reagent probably hydrolyses before it can penetrate very deeply into the more inaccessible pores of the collagen fiber. Indeed, repeated treatment with this reagent modifies only 20–21 of the 33 amino groups on each collagen α chain. On the other hand, treatment with acetic anhydride, ethoxyformic anhydride, and acrylonitrile blocks 100, 92, and 100% of the amino groups, respectively. The greater reactivity of amino groups toward these latter three reagents is probably a result of their smaller size and greater stability in water. The small size would enable them to penetrate small pores, while the greater stability would give them sufficient time to diffuse deeply into the pores. Acetic anhydride, ethoxyformic anhydride, and acrylonitrile can all assume an extended, linear conformation with an unhydrated diameter no larger than an unhydrated phosphate ion—and phosphate ions are known to penetrate collagen fibers of bone and dentin. Citraconic anhydride, a cyclic compound, cannot assume a conformation with a diameter this small, so that many of the lysine/hydroxylysine residues would be inaccessible.

In view of these results, and of the well-known reactivity of aldimines and ketones toward nucleophilic amino compounds, it seems very likely that the transformation of reducible cross-links involves nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of these cross-links, as indicated in Figure 4. Since the only electrophilic atom in reducible cross-links V, VI, and VII is the carbon atom of the double bond, addition of lysine and/or hydroxylysine to these residues must necessarily yield the corresponding products VIIIa, VIIIb, VIIIc, and IX. Cross-links VIIIa, b, and c could join up to three different tropocollagen molecules together. Since they are *gem*-diamines, they have no $NaBH_4$-reducible bonds, but should be labile to hot acids. Cross-link IX could react with an additional lysine or hydroxylysine residue to yield the nonreducible cross-link X, which could join up to four tropocollagen molecules together. This cross-link which, like VIIIa, b, and c, is a *gem*-diamine, should also be cleaved by heat, especially in the presence of acids. Indeed, simple *gem*-diamines, which are synthesized by reacting excess amine with aldimine, aldehyde, or ketone, are not generally stable to distillation unless both amino groups are disubstituted (Smith, 1965). Formation of X could occur by direct displacement of the hydroxyl group of IX, or by dehydration

of IX followed by addition of lysine or hydroxylysine. The dehydration product of cross-link IX probably does not accumulate since no one has reported the $NaBH_4$ reduction product of this reducible cross-link, despite intense activity in this field in recent years.

Formation of cross-links possessing these structures would explain the increasing resistance of maturing collagen fibers to dissolution in cold acid or alkalai, to mechanical disruption, and to thermal disruption. Not only are the nonreducible cross-links inherently more stable than the aldimine cross-links, they are also capable of joining more than two tropocollagen molecules together, whereas V, VI, and VII can join only two molecules. Furthermore, reaction with amino groups would also force any equilibrium between the reducible cross-links and their precursors to favor cross-link formation by the mass law effect.

All the reactions proposed above are perfectly consistent with the known reactivity of amines, aldimines, and carbonyl compounds (Smith, 1965; Layer, 1963). Indeed, cyclic *gem*-diamines form spontaneously when equimolar mixtures of 1,3-diamines are mixed with aldehydes or ketones (Brown, 1962). Furthermore, many plastics such as the urea and melamine resins are created through the formation of *gem*-diamines and analogous compounds. At the present time, formation of cross-links VIIIa, b, and c and IX or X during maturation of collagen fibers appears to be the only acceptable explanation for the data. Certainly, in vivo reduction of the reducible cross-links has been clearly ruled out (Robins and Bailey, 1973; Davis, 1973; Eyre and Glimcher, 1973).

A third type of aldimine has been proposed as a cross-link for soft tissue collagens in addition to V, VI, and VII (Tanzer et al., 1973). However, a more recent study suggests that this aldimine is an artifact which does not exist in vivo (Robins and Bailey, 1974). If it does exist, it might be expected to transform during maturation into a *gem*-diamine analogous to VIII, so modification studies similar to those reported here may be in order.

The temperature dependence of cross-link transformation is quite striking and may provide valuable clues as to the nature of the process. At 3° there is no discernible transformation of reducible cross-links after as long as 11 months. Even at 23°, the rate of cross-link transformation is very slow relative to the rate at 37°. After 70 days at room temperature, the content of reducible cross-links has fallen by approximately 50%, whereas at 37°, 50% of the reducible cross-links have transformed within 10 days. This suggests that the rate-limiting process in cross-link transformation might involve a slow rearrangement of the local collagen structure, necessary for approach of the amino group of lysine or hydroxylysine residues to within attacking distance of the electrophilic double bond of the reducible cross-links. This would be expected if the nucleophilic amino group were on a third collagen molecule. Indeed, realignment of molecules within the initial aggregates may well precede the formation of reducible cross-links, since there is a progressive increase in the content of these reducible cross-links for several hours after initial aggregation. Further, more subtle rearrangements are therefore quite conceivable. The necessity for structural rearrangements prior to transformation would explain the accumulation of reducible cross-links in young, growing fibrils, and the marked insensitivity of transformation to pH. Bovine bone collagen cross-links, for example, transform equally well at pH 5.0, pH 6.0, pH 7.0, and pH 8.5. This insensitivity of transformation rate to pH indicates that nucleophilic attack by lysine or hydroxylysine (which requires deprotonation steps) must occur *after* the rate-determining step—as would be predicted if approach of nucleophilic lysine/hydroxylysine residues, brought about by local structural rearrangements, were rate limiting. The differing rates at which reducible cross-links are transformed in different collagen fibers may also be explained, at least in part, on the basis of a rate-limiting, localized structural change. Indeed, the lateral packing of collagen molecules in bone, dentin, rat tail tendon, and reconstituted collagens is known to differ significantly (Katz and Li, 1972). Whether other factors such as the presence of extracellular molecules and macromolecules can influence the rate of cross-link maturation remains to be seen.

The major reducible cross-links of hard tissue collagens, dehydrohydroxylysinonorleucine and dehydrohydroxylysinohydroxynorleucine, are present predominantly in the keto forms (VIIc, X = H and OH, respectively). Therefore, addition of lysine/hydroxylysine to these cross-links would yield mainly the diamine, IX. Cross-link IX is very likely transformed further, since it should be in dynamic equilibrium with its corresponding aldimine, and, if further transformation of IX were not occurring, $NaBH_4$ reduction of hard tissue collagens would yield the reduction product of this aldimine. No such product has been reported.

If lysine or hydroxylysine residues add to IX, the products will have the *gem*-diamine structure, X. While the modification experiments appear to rule out an essential role for other nucleophilic amino acids in the transformation of known *reducible* cross-links (V, VI, VIIa, and VIIc), their role in transformation of IX remains an open question which is beyond assessment by the present experiments. However, cross-links of general structure IX can only form in collagens where the keto form of reducible cross-links is present. In other collagens, only lysine and/or hydroxylysine residues would appear to be involved in reducible cross-link transformation.

It should perhaps be noted that glutamine and asparagine residues, for which no specific modifications are available, are known to be involved in the chemical cross-linking of proteins by HCHO (Fraenkel-Conrat and Olcott, 1948). In this process, lysine residues react with formaldehyde, and the adduct is then attacked by glutamine/asparagine residues, or by lysine residues, to yield *gem*-diamine analogs, or *gem*-diamines, respectively. That is, HCHO treatment of lysine-containing proteins (and only such proteins) at 20° produces cross-links analogous to the *gem*-diamine cross-links of mature collagen fibers. These observations indicate that weakly nucleophilic glutamine and asparagine residues are capable of forming stable cross-links with aldehydes and lysine, and so must be considered for a role in transformation of IX.

A direct examination of small cross-linked peptides derived from CNBr-solubilized fibers will be necessary to settle the question of whether other nucleophilic residues, as well as lysine/hydroxylysine residues, are involved in collagen cross-linking. It should be possible to isolate multichained peptides joined by the nonreducible cross-links described in this paper. CNBr digestion of insoluble collagen fibers is known to yield soluble peptides, several of which are cross-linked. Maximal yields of peptides containing the nonreducible cross-links should be obtained from the very insoluble collagens of older animals since these collagens contain minimal amounts of reducible cross-links. Alterna-

tively, collagens aged in vitro could be used. For hard tissue collagens, the peptides cross-linked by IX and X can be identified by thermal treatment, which regenerates the easily identifiable cross-links VI and VII. Isolation of such cross-linked peptides will permit a far more detailed study of the nonreducible cross-links of collagen than is currently possible, and may indicate if lysine, hydroxylysine, or both residues are solely responsible for stable, nonreducible cross-link formation during maturation, as we tentatively propose as the simplest explanation currently available.

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