STABLE CROSSLINKS OF COLLAGEN

Norman R. Davis
Dept. of Oral Biology, University of Alberta, Edmonton, Canada.

Received August 15,1973

SUMMARY: It has recently been proposed that the syndesine crosslinks of collagen possess their unique stability because they can isomerize to a α-keto-amine. Although this type of isomerization is perfectly feasible for α-hydroxy-aldimines like syndesine, the present studies suggest that the amount of α-keto-amine present in some tissues is far too low to account for the observed stability. However, it is possible that the syndesine crosslinks adopt a cyclic hydrogen-bonded conformation. This could present considerable steric hindrance to hydration of the C=N bond, rendering this aldimine crosslink much more stable than those with no α-hydroxy group.

INTRODUCTION: It is now well-established that the important physical properties of collagen fibers depend to a large degree on a system of covalent intermolecular crosslinks. All of the known intermolecular crosslinks are aldimines which are formed by the reaction of lysine or hydroxylysine residues on one tropocollagen molecule with lysine or hydroxylysine - derived aldehyde residues on another tropocollagen molecule, as indicated in figure one. Syndesine, the major aldimine crosslink of bone, dentin and cartilage collagens (1,2) has been shown (3,4) to be dehydro-hydroxylysinohydroxynorleucine (Δ^6-HLHNL, I figure one). This crosslink is also one of the three major aldimine crosslinks of tendon, scar tissue, foetal bovine skin and various basement membrane collagens (2,5,6,7), and it appears to be the major aldimine crosslink in the cartilage collagen of cyclostomes and cartilagenous fish, as well as in the collagen of invertebrates (8). Two other aldimine crosslinks (II and III, figure one) have also been found in various collagen tissues. The relative distribution of I, II and III varies with physiological age, species, and type of tissue.

Bailey et. al. were the first to propose an aldimine structure for intermolecular collagen crosslinks, and to actually isolate their $NaBH_4$ reduction products from acid hydrolysates of $NaBH_4$-treated native collagen fibres. These workers also discovered a fundamental difference in the stability of crosslink I as compared to crosslinks II and III. While crosslink I is relatively stable

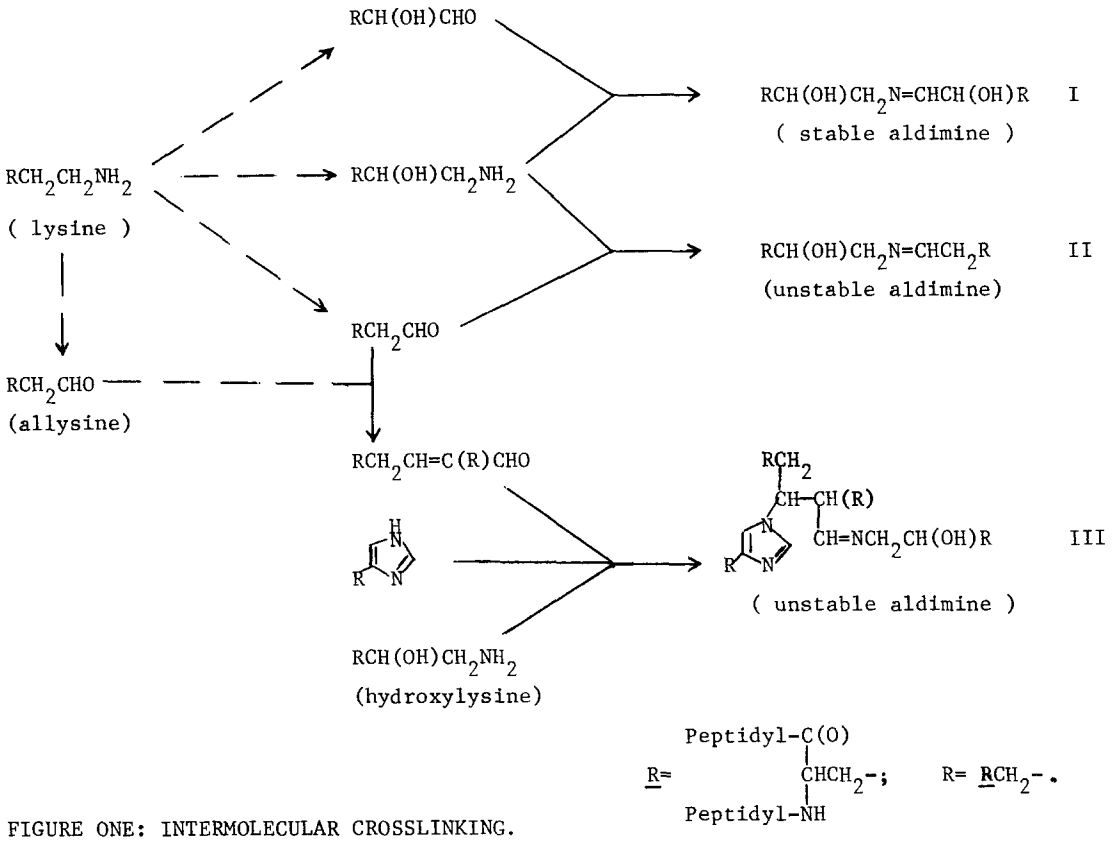


FIGURE ONE: INTERMOLECULAR CROSSLINKING.

to thermal denaturation (2 minutes at 70°), to dilute acids, and to α-amino-β-thiols, crosslinks II and III are extremely labile (2). In view of the importance of crosslink I in collagens from a wide variety of vertebrate and invertebrate tissues and its close structural resemblance to crosslinks II and III, an understanding of the reasons for this stability difference could prove quite useful.

One obvious distinction between the crosslinks is the presence of a hydroxyl group α to the aldimine carbon in I, and its absence from II and III. Because of this hydroxyl group, the α-hydroxy-aldimine structure, I, could be in equilibrium with its α-keto-amine form (Ib, figure two) via the enaminol intermediate. Isomerization of I to Ib could therefore explain the stability of I relative to II and III. However, during synthetic and degradative studies

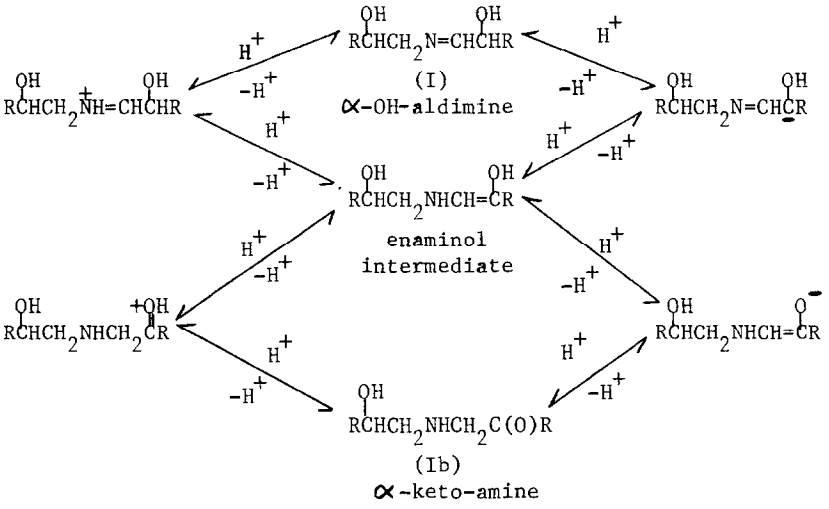


FIGURE TWO: REARRANGEMENT OF α-HYDROXY-ALDIMINE CROSSLINK (I).

on the structure of the [^{3}H]-$NaBH_4$ reduction product of crosslink I (HLHNL, figure three), it was ascertained that at least 95% of this crosslink in calf achilles tendon was in the α-hydroxy-aldimine form, I (ref. 3 and unpublished results of N.R. Davis). Hence, it was felt that α-hydroxy-aldimine/enaminol/ α-keto-amine tautomerization was not the explanation for the stability of I.

Nevertheless, in view of the recent suggestion by Robin's et. al. (6) that isomerization of I is indeed responsible for its stability, it seemed appropriate to determine the relative amounts of I and Ib in a variety of tissues. Indeed, while the present work was in progress, an excellent paper was published by Eyre and Glimcher (9), suggesting that significant amounts of Ib are present in small peptides from calf bone collagen.

METHODS: Native collagen fibres were reduced with [^{3}H]-$NaBH_4$ by two different methods. In addition to the pH 7.4 reduction technique of Bailey et. al. (2), reductions at pH 11 were carried out on collagen samples pre-equilibrated at pH 11 for one hour. After reduction for two hours, the collagen fibres were washed free of salts and hydrolysed in 6N HCl (110°, 22 hr.). Samples (30 mg.) of each hydrolysate residue were chromatographed on an extended basic column

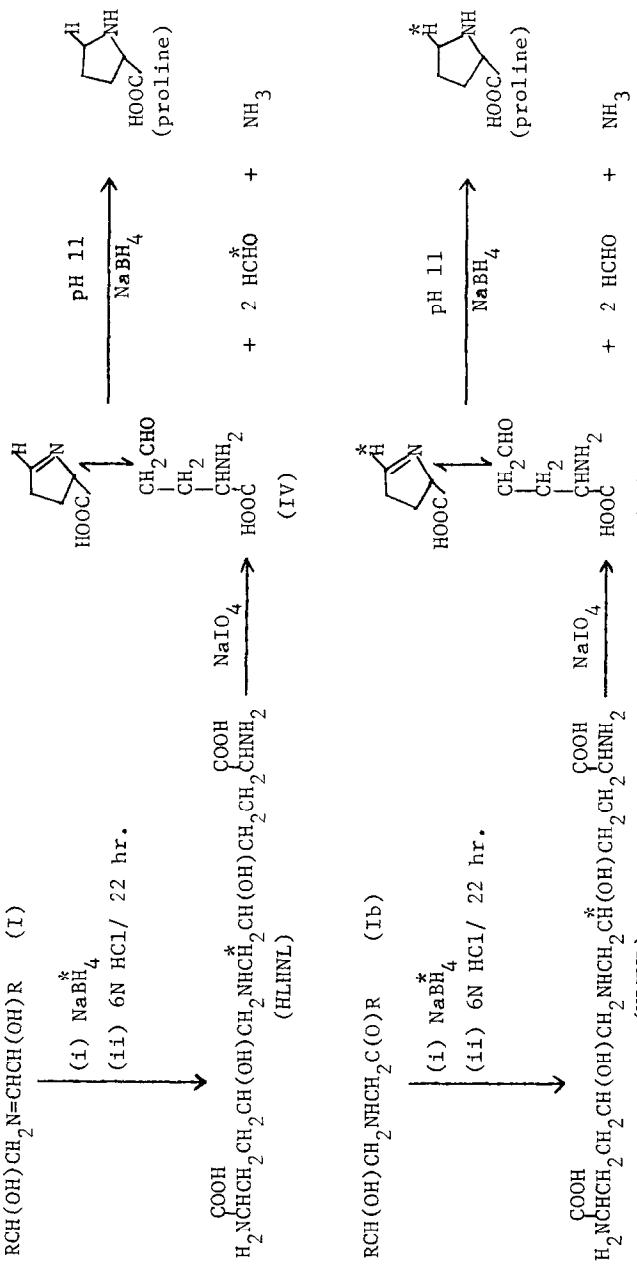
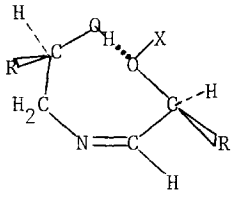


FIGURE THREE: CROSSLINK LABELLING AND DEGRADATION.



X = H or Disaccharide.

FIGURE FOUR: CYCLIC HYDROGEN-BONDED FORM OF Δ^6-HLHNL.

(Beckmann 120B amino acid analyser) as described previously (10). Fractions containing reduced I (HLHNL, figure 3) were combined and desalted on Dowex 50 x 8 columns. After elution with 2M NH_4OH, the tritiated HLHNL from human dentin (50-60 years old) and bovine bone, dentin, and achilles tendon collagens were each studied by the specific degradative techniques described previously (3,11) and summarized in figure three.

Before degradation, each tritiated HLHNL sample was evaporated to dryness six times from 0.1N HCl solutions to remove any exchangeable tritium. Stock solutions were then prepared for determination of specific activity, and aliquots treated with 0.01 M $NaIO_4$ (3,11) to effect oxidation to HCHO, NH_3 and the δ-semialdehyde of α-amino-pentanoic acid (IV, figure three). After two hours, oxidized samples were treated with acidic arsenite to destroy $NaIO_4$, and then divided into equal measured portions. The first portion was distilled to dryness from 15% HCHO solutions six times to remove any tritiated HCHO, and the residue (containing IV, which is non-volatile) dissolved in water for the determination of tritium content. The second portion was reduced with $NaBH_4$ (3,11) to convert IV to proline, as indicated in figure three. This reduction mixture was then chromatographed on a Beckmann 120B analyser and the proline fractions collected for tritium counting (3,11).

RESULTS AND DISCUSSION: Previous work (3,11) has shown that 0.01 M $NaIO_4$ oxidation of HLHNL (figure three) yields two equivalents of HCHO (tritium from carbon six) and one equivalent of NH_3. Both of these products can be isolated and quantitated by distillation. Hence, the ^3H content of carbon six can be

easily measured as [^{3}H]-HCHO. Moreover, reduction of the $NaIO_4$ oxidation mixture with non-tritiated $NaBH_4$ at pH 11 yields carbons one to five as proline (ca. 1.5 equivalents) and δ-hydroxynorvaline. Consequently, any ^{3}H on carbon five will be trapped in these non-volatile compounds and can be measured either as non-distillable ^{3}H or a purified [^{3}H]-proline. Now, [^{3}H]-$NaBH_4$ reduction tritiates I exclusively at C-6 and Ib exclusively at C-5. Therefore, the ratio of non-volatile tritium to volatile tritium (HCHO) from equal quantities of oxidized HLHNL is a measure of the ratio of α-keto-amine crosslink (Ib) to α-hydroxy-aldimine crosslink (I) in the original collagen samples. Table one summarizes the data from a number of such experiments. The data in column one reports the tritium content of non-volatile oxidation products (carbon five tritium) divided by the tritium content of [^{3}H] HCHO (carbon six tritium), while the data in column two represents the tritium content of purified proline (carbon five tritium) divided by the tritium content of [^{3}H]-HCHO.
Under the standard degradation conditions, about 1.5 μmoles of proline are produced for each μmole of HLHNL oxidized. Theoretically, 2 prolines/HLHNL are possible. Therefore, a correction factor of 1.33 (ie., 2/1.5) is applied in column two. For each type of collagen, the estimate of α-keto-amine content in different experiments is quite reproducible. It is clear that in some tissues, very little α-keto-amine is present. Indeed, the low content of Ib in human dentin from middle-aged men is most interesting, since it indicates that isomerization of I to Ib can be very slow.

It is difficult to see how isomerization of I could be responsible for the stability of these crosslinks, in view of the observation (N.R. Davis, unpublished results) that I is just as stable in tissues with very little α-keto-amine as in tissues with significant amounts of α-keto-amine. For example, the Δ^6-HLHNL (I) content of human dentin (age 11-70 years) is not altered by heating in physiological buffer for three hours at 70° or for one hour at 90°, in spite of the almost negligible α-keto-amine content.

Since aldimine-enamine tautomerization and keto-enol tautomerization are

Collagen Source	$\frac{\text{Non Volatile } {}^3H}{\text{Volatile } {}^3H}$	$1.33 \times \frac{{}^3H \text{ Pro}}{\text{Volatile } {}^3H}$	% α-keto-amine
Bovine Bone+	0.43	Not Done	30 ± 3%
Bovine Bone*	0.45	0.47	31 ± 3%
Bovine Dentin+	0.25	Not Done	20 ± 3%
Bovine Dentin*	0.27	0.31	22 ± 3%
Bovine Tendon*	0.04 ± 0.07	Not Done	4 ± 1%
Human Dentin* 51-60 yr.	0.05	0.03	4 ± 1%

+Equilibrated and reduced at pH 11
*Equilibrated and reduced at pH 7.4

TABLE ONE: PROPORTION OF Δ^6-HLHNL IN α-KETO-AMINE FORM

catalysed markedly by acids and bases, isomerization of I and Ib could be base-catalysed. Interestingly, pretreatment with 10^{-3} M NaOH and reduction with basic [^{3}H]-$NaBH_4$ gave essentially the same estimates of α-keto-amine content as did reduction of collagen at pH 7.4. It is possible that Δ^6-HLHNL adopts a conformation which, in some tissues at least, presents a stereo-electronic barrier to isomerization even in the presence of catalyst. There are numerous examples in the literature of compounds which are frozen into conformations whose geometry prevents the attainment of transition states essential for certain reactions (see, for example, H.O. House, ref. 12). Hence, even under drastic conditions, they cannot undergo reactions which occur readily in other conformations. It is an open question whether all the α-keto-amine is localized at one particular crosslink position (for example, the glycosylated peptide isolated by Eyre and Glimcher, ref. 9), or whether it is produced at a number of positions. An analogous reaction, the Amadori rearrangement, certainly requires catalysis, so the presence or absence of adjacent acidic or basic residues could be critical to the production of Ib from I (acid or base catalysis).

Since the α-hydroxy-aldimine seems to be the major form of Δ^6-HLHNL in all tissues studied, one must explain how this aldimine resists hydration by

water and subsequent cleavage to hydroxylysine and hydroxyallysine at 70°. A study of CPK molecular models of I has indicated that the syn form of this aldimine can adopt a cyclic, hydrogen-bonded structure (figure four) which is flexible enough to permit several similar conformations. On the other hand, the cyclic anti isomer of I is inflexible, somewhat strained, and probably does not form. Model building indicates that Δ^6-HLHNL crosslinks with a covalently bound disaccharide (X, figure four) can also adopt the cyclic structure, provided the glycosidic oxygen acts as H-acceptor in the hydrogen bond. What is especially striking about most of these conformers is the moderate to severe steric hinderance to approach of water at the C=N bond. If tropocollagen packing forces and the cyclic hydrogen-bonding interaction diagramed in figure four effectively freeze I into a conformation which resists attack by water, this crosslink should be rather stable at neutral pH.

Whether or not adoption of a cyclic structure is responsible for the stability of Δ^6-HLHNL crosslinks in collagen, the experiments described above indicate that the α-keto-amine content of collagen is too low to account for such stability. It appears therefore, that attention might best be focused on the α-hydroxy-aldimine form of this crosslink in our efforts to solve the mystery of the age-related disappearance of Δ^6-HLHNL from the collagen of numerous species and tissues (6,13). Chemical considerations suggest that this process could involve a slow addition of some nucleophilic species (such as lysine or histidine) across the C=N bond to yield a non-reducible but thermally labile crosslink. The rate-limiting process might involve a slow rearrangement of the local collagen structure, necessary for approach of the presumed nucleophilic to within attacking distance from the C=N band. Such a process could well proceed more quickly with purified fibres in vitro, as has been observed by this author and others. At high temperatures, thermal denaturation could rupture the non-reducible band, regenerating I and withdrawing the nucleophile. This would explain the thermal reversibility of the "aging" process in some tissues (N.R. Davis, unpublished results) as well as the aging process itself.

ACKNOWLEDGEMENTS: The Author wishes to thank the Medical Research Council of Canada for financial assistance, and Dr. L.B. Smillie for generous use of his auto-analyser.

REFERENCES

1. Bailey, A.J., Fowler, L.J., and Peach, C.M. (1969), Biochem. Biophys. Res. Commun., 35, 663.
2. Bailey, A.J., Peach, C.M., and Fowler, L.J. (1970), Biochem. J. 117, 819.
3. Davis, N.R., and Bailey, A.J. (1971), Biochem.Biophys.Res.Commun., 45, 1416.
4. Mechanic, G., and Tanzer, M.L. (1970), Biochem.Biophys.Res.Commun., 41, 1597.
5. Forrest, L., Shuttleworth, A., Jackson, D.S., and Mechanic, G.L., Biochem. Biophys. Res. Commun. (1972), 46, 1776.
6. Robins, S.P., Shimokomaki, M., and Bailey, A.J. (1973), Biochem. J., 131, 771.
7. Tanzer, M.L., and Kefalides, N.A. (1973), Biochem.Biophys.Res.Commun., 51, 775.
8. Bailey, A.J. (1971), FEBS Letters, 18, 154.
9. Eyre, D.R., and Glimcher, M.J. (1973), Biochem.Biophys.Res.Commun., 52, 663.
10. Davis, N.R. (1973), Biochem. Biophys. Res. Commun., 52, 877.
11. Davis, N.R., and Bailey, A.J. (1972), Biochem. J., 129, 91.
12. House, H.O., "Modern Synthetic Reactions" (1965), W.A. Benjamin Inc., New York, Amsterdam.
13. Bailey, A.J., and Skimokomaki, M.S. (1971), FEBS Letters, 16, 86.