# A METHOD FOR THE DETERMINATION OF LYSINE-DERIVED COLLAGEN CROSSLINKS

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SUMMARY: A rapid and inexpensive method was devised for the determination of lysine-derived aldimine crosslink contents in collagen. The aldimines were converted to their secondary amine derivatives by $NaBH_4$ reduction, and the acid or base collagen hydrolysates analysed directly for these derivatives (HLHNL and HLNL). It was found that in native bone, dentin and cartilage collagen fibres, every two tropocollagen molecules are joined by a minimum of one aldimine crosslink. Negligible amounts of HLNL and HNHNL were found in unreduced collagens, indicating that maturation does not involve a simple in-vivo reduction of the aldimine crosslinks.

INTRODUCTION: In the last five years several interesting papers have been published on the lysine-derived crosslinks which appear to play such an important role in the stability, maturation, and physiochemical properties of native collagen fibres. Bailey et al (1,2) were the first to demonstrate the presence of acid-labile, $NaBH_4$-reducible intermolecular crosslinks in native collagen fibres, and postulated for them an aldimine-type of structure derived from lysine residues. This group then isolated the $NaBH_4$-reduction products of three aldimine cross-links, and elucidated the structure of II (figure one). The major crosslink of bone and dentin was later shown to be I (figure one) by direct chemical synthesis, confirmed by chromatographic, mass spectral, and degradative studies (3). Tanzer and Mechanic (4) have recently confirmed the presence of these crosslinks in numerous collagen tissues pretreated with $NaBH_4$. Indeed, their mass spectral studies first indicated the probable structure of I. The probable biosynthetic routes to I and II are indicated in figure one.

Recent studies by Bailey (5) indicate that in rat, bovine and human soft tissue collagens, aldimines I and II gradually disappear as the animals reach maturity. Since these changes are virtually complete by maturity (4-5 years in bovine soft tissues, and 17-20 years in human tissues), they appear to be a normal part of the growth process rather than an aging process. Bailey et al therefore consider I and II to be intermediates on the way to more complex crosslinks of unknown structure.

Mechanic et al (6) have recently suggested that I and II may be reduced in vivo to their respective secondary amine derivatives (HLHNL and HLNL) in both bone and dentine collagen, and that the relative proportions of I, II, HLHNL and HLNL varies with the age of the animal and with the type of tissue studied.

In view of the fact that no simple methods were available for estimating the absolute (as opposed to relative) content of I, II, HLHNL and HLNL in collagen, the present studies were undertaken. The convenient and inexpensive methods developed have allowed a quantitative estimate of I, II, HLHNL and HLNL contents to be made for several hard and soft tissue collagens for the first time, with some important implications.

METHODS: All collagens were prepared by conventional procedures, except that dentin collagens were extracted with tris-NaCl to remove phosphoprotein (7). Native bone, dentin and soft tissue collagens were reduced with $NaBH_4$ as described by Bailey et al (2), except that a 2 hour reduction period was employed Reduced and unreduced collagen samples were hydrolysed for 22 hours each, using constant boiling HCl under $N_2$ at $110 \pm 1^{o}$. Several different amino acid analysers of varying design and sensitivity were employed, as indicated below. In order to avoid overloading and unsatisfactory baselines, "unfractionated hydrolysates" were not pumped into the ninhydrin reaction coil until all the acidic and neutral aliphatic amino acids had emerged from the analyser columns. A stable baseline was obtained by pumping ninhydrin and buffer from a second column through the coil, and the column eluents were manually switched from drain to coil just before emergence of tyrosine (except in the case of the technicon amino acid analyser, where HLHNL and HLNL emerge before tyrosine).

For analysis of large amounts of collagen hydrolysates, the acidic and neutral aliphatic amino acids were first removed by fractionation on a CGC-240 column (2.6 x 31 cm) using 0.38M sodium citrate buffer (pH 4.30) at $23^{o}$. The resin was freshly regenerated with 130 - 150 ml of 0.2M NaOH, followed by 30 ml of water and 130 ml of 0.38M citrate (pH 4.30). Collagen hydrolysates

(0.1 to 1 gm) were applied in pH 2.2 citrate and washed in with the same buffer. The columns were then eluted with 380 ml of 0.38M citrate (pH 4.30) at 55 ml per hour. Next, the columns were washed with 150 ml of $H_2O$ to remove salts (a negative Ag $NO_3$ test for $Cl^-$ ions) and the HLNL, HLHNL, posthisdine fraction, aromatic and basic amino acids co-eluted with 200 ml of 2M $NH_4OH$. The residue left after evaporation of the $NH_4OH$ contained about ten times the concentration of HLHNL and HLNL as compared to the unfractionated hydrolysate from $NaBH_4$-reduced collagens.

All amino acid analysers were calibrated with synthetic HLHNL (8) and HLNL (2), and operated as described below.

Technicon (125 cm Chromosorb A Column, 60°): CGC-fractionated hydrolysates from over 200 mg of collagen can be analysed on this system. HLHNL and HLNL emerge at 96 and 108 minutes, while Tyr and Phe do not emerge until 126 and 152 minutes, respectively. The developing buffer (0.8 M $Na^+$, pH 5.00 citrate) is pumped at 30 ml. $hr.^{-1}$ in this system, which can easily detect 0.01 umoles of HLHNL or HLNL.

Jeol JLC-5AH (51 cm column at 60°): Development of this column with 0.35 M $Na^+$ (pH 5.28 citrate) at 48 ml $hr.^{-1}$ (9) elutes tyr, phe, HLHNL and HLNL at 55, 64, 85, and 104/108 (double peak) minutes, respectively. Amino acids eluting before 40 minutes were sent to drain. 1-2 mg collagen samples were analysed with the high sensitivity colorimeter cell, and 6-10 mg of unfractionated collagen hydrolysates were analysed with the lower sensitivity cell.

Beckmann 120B (55 cm. A50 column, 56°): Amino acids from 10-20 mg of hydrolysed collagen were eluted with pH 5.28 (0.35 M $Na^+$) buffer at 70 ml. $hr^{-1}$. The first 28 ml. (25 min.) contained neutrals and acidics, and were sent to drain. Tyrosine, phe, HLHNL, HLNL and his emerged at 40, 45, 60, 70 and 160 minutes, respectively. Any extended basic column should serve equally well. Indeed, both Beckmann and Locart basic columns have been used previously in the purification of natural and synthetic HLHNL (3,8).

On the 55 cm. neutral column of this analyser, HLHNL gave interesting

but non-integrable double peaks with 0.38 M (pH 4.26) buffer as well as the above buffer, indicating for the first time the mixture of isomers expected for this compound.

RESULTS AND DISCUSSION: The problems associated with detection of aldimine cross-links I and II revolve around their very low content relative to other amino acid residues in collagen, as well as their instability to extremes of temperature and pH. As aldimines, they are necessarily cleaved into aldehyde and lysine fragments by aqueous acids and bases, and so cannot survive hydrolysis of collagen fibers. However, they can be isolated (2) as their $NaBH_4$ reduction products (HLNL and HLHNL, figure one), which are stable to 6N HCl.

In the past, I and II have been detected by conversion to the radioactive reduction products (HLNL and HLHNL) with $^3H$ - $NaBH_4$. Although this allowed an estimate of the relative proportion of HLNL: HLHNL, estimates of the absolute content (residues per $\alpha$ chain) are difficult. Furthermore, such procedures are error-prone, time-consuming and expensive.

If I and II are the only important reducible crosslinks in bone and dentin collagens, it was reasoned that they should, collectively, amount to at least 0.17 residues per $\alpha$-chain (one crosslink between each 2 tropocollagens). Therefore, it seemed reasonable to assume that if enough collagen were reduced and hydrolysed, the reduction products (HLNL and HLHNL) would be detectable on most amino acid analysers. Bailey et al (2) had shown that HLNL and HLHNL were separable from the other amino acids of collagen. Therefore, it appeared that the major problem in direct analysis of collagen hydrolysates for HLNL and HLHNL would be to avoid overloading amino acid analysers, since this would result in unstable baselines and even precipitation of ninhydrin in the reaction coil. Two solutions to this problem were found.

For smaller amounts of collagen and reduced collagen samples (1 to 20 mg), overloading was avoided by sending all the eluted acidic and neutral aliphatic amino acids to drain, and manually switching the solvent to the ninhydrin reaction coil just before the emergence of tyrosine. This gave a stable base-

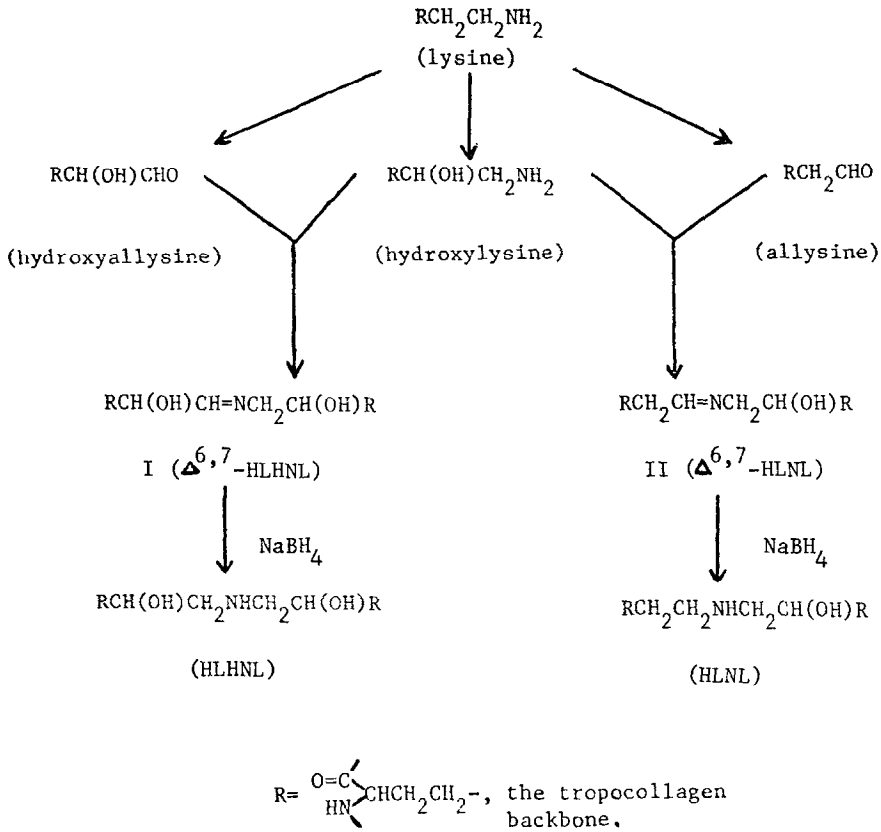


FIGURE ONE: FORMATION OF LYSINE-DERIVED CROSSLINKS, I AND II.

line, and enabled relatively large amounts of collagen (over 20 mg) hydrolysate to be analysed directly for HLNL and HLHNL. Pumping the acidic and neutral amino acids to drain is important even for analysis of 1 mg of collagen hydrolysates on sensitive analysers such as the Jeol 5AH, since failure to do so often gives unsatisfactory baselines and crosslink peaks which are impossible to integrate quantitatively.

Pre-fractionation of collagen and reduced collagen hydrolysates on CGC resin removes 90% of the amino acids. Hence, ten times as much collagen can be analysed for crosslinks without overloading the analyser columns. This is especially useful for less sensitive analysers. Moreover, it allows detection of extremely low amounts of crosslinks (see below).

Phenylalanine, lysine and histidine serve as ideal internal standards

| Species/Tissue | Aldimine Crosslinks (residues/1000) | | |
|---|---|---|---|
| | I | II | I + II |
| *Bovine Skin | 0.01 (5) | 0.16 | 0.17 ± 0.01 |
| *Bovine Cartilage | 0.13 | 0.06 | 0.19 ± 0.01 |
| *Bovine Bone | 0.23 | 0.06 | 0.27 ± 0.01 |
| *Bovine Dentin (Unerupted) | 0.16 | 0.03 | 0.19 ± 0.01 |
| *Bovine Dentin (Erupted) | 0.16 | 0.03 | 0.19 ± 0.01 |
| Human Dentin, 11 - 13 yr. | 0.22 | 0.04 | 0.26 ± 0.01 |
| Human Dentin, 18 yr. | 0.17 | 0.03 | 0.20 ± 0.01 |
| Human Dentin, 31 - 40 yr. | 0.16 | 0.02 | 0.18 ± 0.01 |
| Human Dentin, 41 -50 yr. | 0.14 | 0.02 | 0.15 ± 0.01 |
| Human Dentin, 51 - 60 yr. | 0.13 | 0.01 (5) | 0.14 ± 0.01 |
| Human Dentin, 61 - 70 yr. | 0.13 | 0.003 | 0.13 ± 0.01 |

*Unknown Age

TABLE I: ALDIMINE CROSSLINK CONTENT OF COLLAGENS

for the determination of HLNL and HLHNL, since the ratio of crosslink peak area to the area of these three peaks is easily determined, and the content of these residues is well-established for most collagens.

The current studies indicate that unreduced collagens contain only traces of HLHNL and HLNL (0 to 0.006 res./1000). Even dentin from human patients up to 70 years old is devoid of significant amounts of these reduced aldimines. It would therefore appear that *in vivo* reduction is not responsible for the almos complete transformation of aldimine crosslinks during maturation. Although Mechanic et al (6) suggested that 25 to 50% of the aldimine crosslinks in dentin and bone may be reduced *in vivo*, they were careful to point out the "potential weaknesses" and "pitfalls" inherent in their methodology. The current studies further underline the danger of relying solely on radioisotope studies and mass spectroscopy when dealing with collagen crosslinks. However, they support Bailey's proposal that I and II are converted to more complex crosslinks during maturation (5).

Collagens treated with $NaBH_4$ to reduce aldimines I and II to HLHNL and HLNL all contain at least one residue of HLHNL + HLNL for each two tropocollagen molecules. As indicated in table one, the content of aldimine crosslinks depends on the age, species and type of collagen tissue examined. Since it is not

known whether I and II are quantitatively converted to HLHNL and HLNL by $NaBH_4$ reduction, the values in table I represent a minimal crosslink content.

Time studies (22 to 120 hours) on reduced collagen fibres, and on pure synthetic HLHNL, indicate that HLHNL is surprisingly stable to the hydrolysis conditions, with less than 5% destruction even after 5 days in 6NHCl at $110 \pm 1^{\circ}$. However, if the aldimine crosslinks are estimated by the $^3H$ content of their $^3H$-$NaBD_4$ reduction products, caution should be used, since hydrolysis could effect the $^3H$ content, as it does for many radioactive amino acids. This is a further drawback to previous methods of aldimine crosslink estimation.

It is interesting to note that in dentin collagen, there is relatively little disappearance of the aldimine crosslinks during maturation, in contrast to the almost complete disappearance of such crosslinks from soft tissues. However, during aging (21 to 70 years), a 20-25% decrease in these crosslinks is observed. This could be the result of a true aging process, the formation of secondary dentin with a lower content of aldimine crosslinks, or both. Further studies on the absolute content of aldimine crosslinks in a variety of tissues of different ages and physiological states could prove most interesting, and should be aided considerably by the techniques described above.

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