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THE HYDROXYPYRIDINIUM CROSSLINKS OF SKELETAL COLLAGENS: THEIR MEASUREMENT, PROPERTIES AND A PROPOSED PATHWAY OF FORMATION

David R. Eyre+ and Haruhisa Oguchi*

Harvard Medical School and Orthopaedic Research Laboratories
Children's Hospital Medical Center, Boston, MA 02115

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A method is described for quantifying both reducible and mature crosslinking amino acids of collagen. The main crosslinking residue in cartilage, dentine and mature bone was the 3-hydroxypyridinium compound identified by Fujimoto et al. (1-3). Adult articular cartilage contained about one residue per collagen molecule, over forty times the content of the reducible crosslinks. We propose that hydroxypyridinium residues are formed by spontaneous interaction of two residues of hydroxylysino-5-ketonorleucine. This reaction explains the disappearance of reducible crosslinks at maturity and provides a novel mechanism for lateral crosslinking within and between fibrils which may account for some of the unique physical properties of hard tissue collagens.

Collagen is the principal structural protein of animals. Its strength depends on the formation of covalent bonds between molecular monomers packed in fibrils. Most of the known crosslinking residues are aldimines or related structures that were isolated and identified only after chemically reducing them in the protein with $NaBH_4$. They gradually disappear from adult tissues but the modifying reactions and their mature products are unknown (4).

Recently a fluorescent crosslinking amino acid was isolated from collagen of ox tendon and bone and shown by NMR spectroscopy and other techniques to be a 3-hydroxypyridinium derivative that had three amino acid side-chains (1,2). While developing a method for quantifying crosslinking amino acids of cartilage collagen, we isolated a similar fluorescent compound in high yield. Its abundance in various skeletal tissues relative to the reducible crosslinking amino acids was measured.

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*Present address: School of Dentistry, Tokyo Medical and Dental University, Bunkyo-Ku, Tokyo, Japan.
+To whom correspondence should be addressed.

ABBREVIATIONS: HylOHNle, hydroxylysinohydroxynorleucine
HylNle, hydroxylysinonorleucine

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MATERIALS AND METHODS:

Bovine articular cartilage came from knee joints of animals at various ages. Cortical bone and dentine were prepared from a 2 year-old steer and cortical bone, articular cartilage and fibrocartilage from one year-old rabbits. Cortical bone and neck tendons were taken from 14 week-old chickens. Powdered bone and dentine were decalcified in 0.5M EDTA, pH 7.5 at 4°C. Some tissue samples were reacted with [^{3}H]$NaBH_4$ (10 Ci/mole) in 0.1M phosphate, pH 7.4 (5). Samples of reduced and non-reduced tissue were hydrolyzed in 6N HCl at 108°C for 24 hours under N_2 in sealed glass tubes.

Molecular Sieve Chromatography: Dried hydrolysates (200 mg) were redissolved in 2 ml of 1M $CaCl_2$, 0.05M Tris/HCl, pH 7.5 and eluted in this buffer from a column (2.5 cm x 90 cm) of Bio-Gel P2, 100-200 mesh (Bio-Rad Labs). Effluent fractions were analyzed for relative fluorescence and tritium activity.

Fractions of interest were pooled, freeze-dried and desalted by elution in 0.1M acetic acid from a shorter column (2.5 cm x 40 cm) of Bio-Gel P2 (200-400 mesh), monitoring effluent for absorbance at 230 nm. Effluent from Vo to immediately before salt was pooled and dried for amino acid analysis.

Amino Acid Analysis: Desalted fractions were routinely assayed on the Beckman 121M amino acid analyzer using a standard 90 minute program for complete analysis that employed a single column eluted stepwise with three buffers. A laboratory-built analyzer previously calibrated for known crosslinking amino acids (5) was also used, eluting the column (50 cm x 0.9 cm) with a single buffer, 0.2N Na citrate, pH 4.42, at 50°C. Crosslinking amino acids were quantified as leucine equivalents and related to the collagen content of the tissue determined by hydroxyproline analysis.

RESULTS:

For all the tissues a single, early peak of fluorescent material eluted from the P2 column run in $CaCl_2$ (Fig. 1). The uncorrected excitation and emission spectra showed $\lambda_{Ex\ max}$ at 330 nm and $\lambda_{Em\ max}$ at 390 nm. When borohydride-treated tissue was analyzed, HylOHNle and HylNle were found to have co-eluted with this fluorescent compound (Fig. 2). Neither of these latter crosslinks was detected in bone or cartilage without borohydride treatment. The recovery of the fluorescent compound was the same, however, whether the tissues were reduced or not. Only traces of amino acids other than these three crosslinking compounds were detected in the fractions under the fluorescent peak (Fig. 3). The fluorescent substance itself eluted on the amino acid analyzer as a single ninhydrin-positive peak, the elution position moving from near leucine to well after phenylalanine on lowering the pH from 5.3 to 4.4. This behavior and the fluorescence spectra are entirely consistent with the properties of pyridinoline, the 3-hydroxypyridinium compound characterized

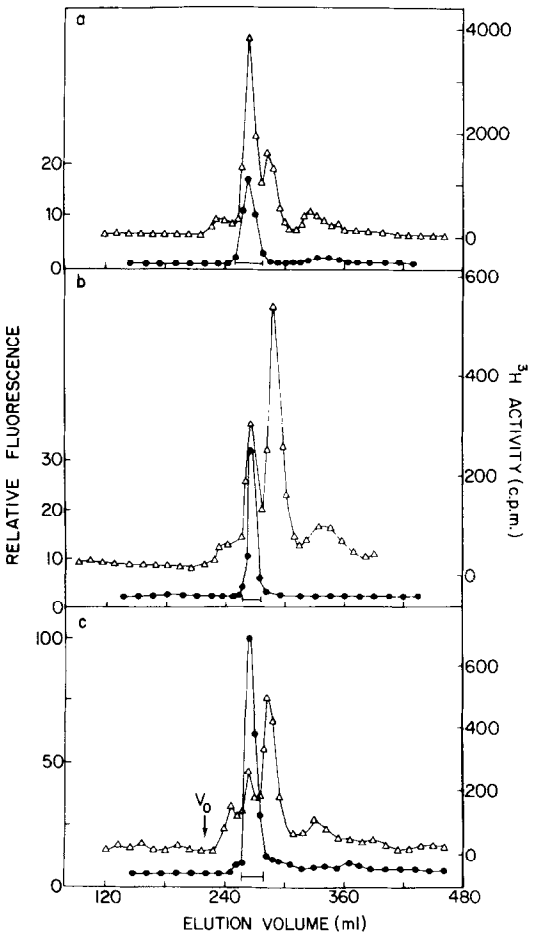

Fig. 1: Molecular sieve chromatography of acid hydrolysates of bone and cartilage collagens on Bio-Gel P2. Tissue was reacted with tritiated $NaBH_4$ before hydrolysis. Effluent fractions were assayed for relative fluorescence (λ_{Ex} = 330, λ_{Em} = 390; ●——●) and tritium activity (Δ——Δ). 200 mg dry weight of each of the following were run:

a) Decalcified rabbit bone
b) Decalcified cow bone
c) Cow articular cartilage

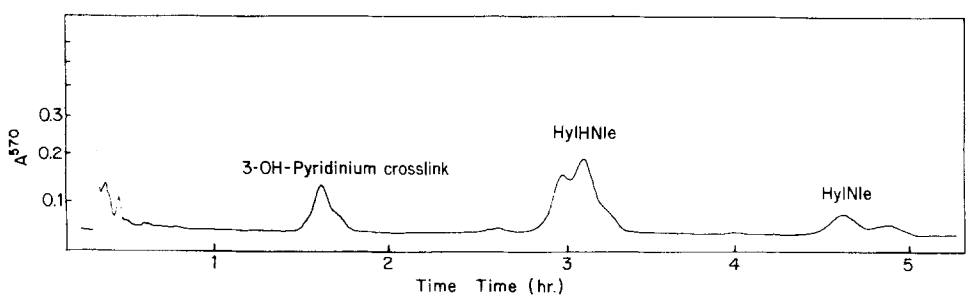

Fig. 2: Amino acid analysis of material recovered from the fractions indicated by the bar in Figure 1a. The column (60 cm x 0.9 cm) was eluted with a single buffer (0.2N sodium citrate, pH 4.42) at 50°C. The absorbance profile at 570 nm after reaction with ninhydrin is shown. Measurements on unreacted column effluent showed that the only significant peak of fluorescence in the chromatogram co-eluted precisely with the peak labelled 3-OH-pyridinium crosslink.

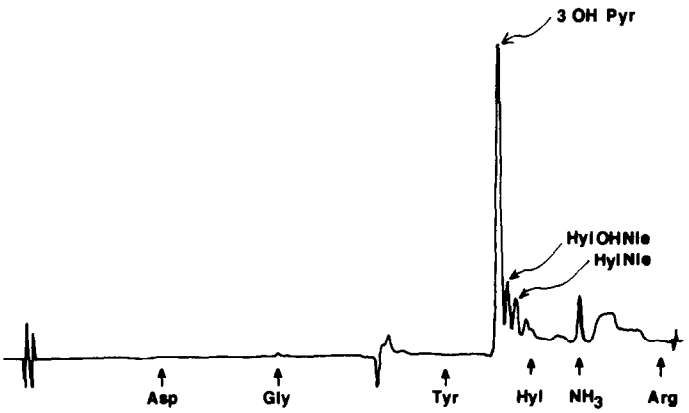

Fig. 3: Amino acid analysis on the Beckman 121M analyzer of material recovered from the fractions indicated by the bar in Figure 1b. The absorbance profile at 570 nm is shown. The three crosslinking compounds are essentially the only amino acids present. Their identity and relative amounts were confirmed by column chromatography of another portion using the method in Figure 2.

by Fujimoto et al. (1,3). On the long column at pH 4.4 the compound always showed characteristic leading and trailing shoulders (Fig. 2) suggesting the presence of stereo-isomers.

The pre-crosslinking peak (230-260 ml, Fig. 1) contained small amounts of ninhydrin-positive substances that were tentatively identified by their elution position on the amino acid analyzer as hexosyllysine and hexosylhydroxylysine which accumulate in collagenous tissues, especially cartilage, with age (4). The identity of the material responsible for the peak of radioactivity eluting immediately after the crosslinking peak (280-320 ml, Fig. 1) is unknown. Lysine and hydroxylysine were virtually the only amino acids present in this fraction. Small amounts of reduced crosslinking aldehydes were detected but not enough to account for all the tritium activity. The following fraction (320-360 ml) contained mainly arginine, and only after this did the rest of the amino acids in the hydrolysate begin to elute.

The content of the hydroxypyridinium crosslink in bovine articular cartilage rose with age to a maximum in the 2 year-old, mature animal. Adult cartilage collagen contained 4-5 times as much as adult bone collagen (Table 1). Body-wall collagen of the sea cucumber, *Thyone briareus*, was

Table 1

CONTENT OF HYDROXYPYRIDINIUM CROSSLINKS IN COLLAGENS OF VARIOUS SKELETAL TISSUES

		Residues of leucine equivalents per collagen molecule*
	OX	
Articular Cartilage	Foetus	0.7
	Calf	1.1
	2 year-old	1.9
	6 year-old	1.5
Cortical Bone	Adult	0.5
Dentine	Adult	0.3
	RABBIT	
Articular Cartilage	1 year-old	1.5
Fibrocartilage (knee meniscus)	1 year-old	0.9
Cortical Bone	1 year-old	0.2
	CHICKEN	
Cortical Bone	14 week-old	0.09
Tendon (Neck)	14 week-old	0.04
	INVERTEBRATE	
Thione briareus Body Wall (Sea Cucumber)		0.9

* Determined by amino acid analysis of the fluorescent fraction isolated by molecular sieve chromatography (Fig. 1). Although the accurate color factor of the hydroxypyridinium compound with ninhydrin is unknown, the absolute number of residues per collagen molecule will be between half and a third the above values.

also rich in it, indicating a widespread distribution in vertebrate and invertebrate species. The ratio of reducible crosslinks to the hydroxypyridinium compound was readily measured by amino acid analysis of the crosslinking fraction isolated from borohydride-treated tissue. The findings are shown in Table 2. Assuming that the molar color yield with ninhydrin for the 3-hydroxypyridinium compound is between two and three leucine equivalents, adult cartilage contains about one residue of the amino acid per molecule of type II collagen. The number of residues of HylOHNle plus HylNle was less than one fortieth of this amount. In bone collagen the proportion of reducible crosslinks was higher (Table 2), the relative amounts of reducible and mature crosslinks apparently being related to age and species. Thus in one year-old rabbit bone, reducible crosslinks still dominated hydroxypyridinium crosslinks by about eight residues to

Table 2

RELATIVE CONTENTS OF REDUCIBLE CROSSLINKS AND THE HYDROXYPYRIDINIUM DERIVATIVE IN BONE AND CARTILAGE COLLAGENS

	Residues of leucine equivalents per collagen molecule		
	OH Pyr.	HylOHNle	HylNle
Cow articular cartilage (2 year-old)	1.9	<.05	not detected
Cow bone (2 year-old)	0.46	0.10	0.08
Rabbit bone (1 year-old)	0.18	0.75	0.25

one, whereas in two year-old cow bone the non-reducible residue had become the major compound.

DISCUSSION:

The findings show that the 3-hydroxypyridinium derivative is the major crosslinking residue in collagen of mature skeletal tissues and is especially abundant in cartilage. Much lower levels were reported for cartilage and bone of the seven week-old rat (3), indicating that its rate of formation is a function of the actual age of the fibrils, not the biological age of the animal. In human cartilage after maturity the content of the fluorescent residues fell with age suggesting modification to yet further structures (6).

Fujimoto et al. (2) showed that the 3-hydroxypyridinium derivative has amino acid side chains at the 1, 4 and 5 ring positions and proposed that it derives by condensation of two hydroxylysyl aldehydes and a hydroxylysine residue. From considerations of chemistry (7), molecular packing and biological function we now propose that this crosslink forms by spontaneous interaction of two difunctional crosslinks and that it has the structure shown in Figure 4. This mechanism of formation has important implications for the properties of collagen fibrils allowing a second, slow stage of intermolecular crosslinking. It explains why the reducible, keto-amine crosslinks disappear from skeletal connective tissues with age, and indicates

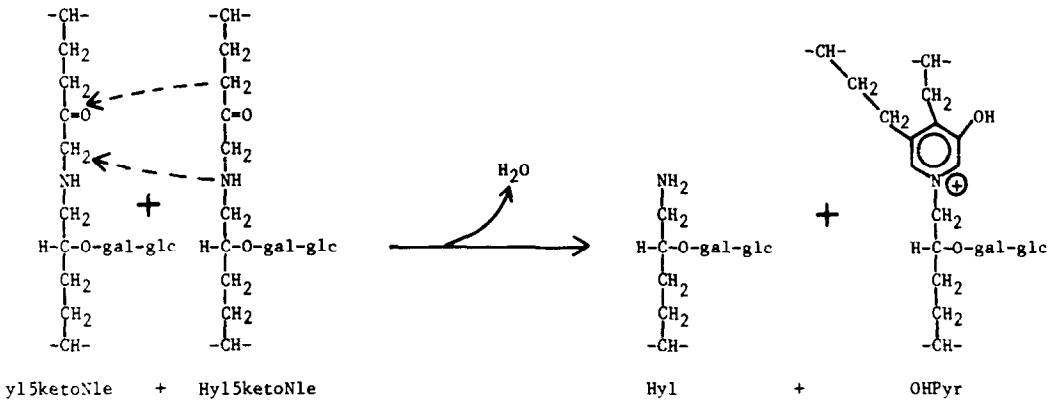

Fig. 4: Proposed natural derivation of the hydroxypyridinium residues in collagen by interaction of reducible crosslinks. This type of condensation reaction is favored chemically (7). Residues of dehydro-HylOHNle in bone and cartilage mainly have the above keto-amine arrangement (4). We predict that chemical reaction between adjacent reducible crosslinks is facilitated by the spatial packing arrangement of collagen molecules and the local environment at the crosslinking site. The precursor crosslinks are shown glycosylated only because most of them are in bone and cartilage (10; Eyre, unpublished).

that the hydroxypyridinium product is one of the long anticipated 'mature', non-reducible crosslinks of collagen.

The possibility that the crosslink was produced artifactually during acid hydrolysis was ruled out by detecting its characteristic fluorescence spectrum in pepsin-solubilized type II collagen and in peptides prepared from bone and cartilage collagens by digestion with bacterial collagenase or CNBr (unpublished observations). Moreover, incorporation of radiolabelled lysine into cartilage in vivo confirmed that the compound derives from lysine and that hydroxylysino-5-ketonorleucine behaves as an intermediate in its formation (Eyre et al., in preparation).

It has been speculated that the extreme insolubility and failure of skeletal tissue collagens to swell in dilute acid may be due to the presence of this crosslink (8). The condensation reaction of reducible crosslinks could also provide a specific mechanism for fibrils to crosslink to each other wherever they make contact in the matrix. This would be especially important in cartilages where it could explain some of the age-related changes, such as the decreasing extractability of proteoglycans (9).

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