Cross-linking of collagen

Location of pyridinoline in bovine articular cartilage at two sites of the molecule

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The location of pyridinoline in 18-month-old bovine articular cartilage was investigated by fractionation of CNBr-derived peptides by ion-exchange chromatography and gel filtration. Two peptides, PCP1 and PCP2, were isolated and were shown to contain stoichiometric amounts of pyridinoline. From its amino acid composition and sequence studies, peptide PCP1 was shown to comprise two C-terminal non-helical chains (CB14) linked through pyridinoline to the $\alpha 1(\text{II})$ -CB12 portion of the helix. The CB14 chains appeared to be labile at their C-terminal ends, resulting in lower-than-expected amounts of homoserine, and only the N-terminal portion of the peptide was sequenced. Similar studies of peptide PCP2 showed that it contained two N-terminal non-helical chains (CB4) linked to the $\alpha 1(\text{II})$ -CB9,7 portion of the helix. The isolated peptides therefore confirmed the function of pyridinoline in stabilizing the 4D stagger of adjacent molecules. The possibility that the cross-link could act both as an intra- and an inter-microfibrillar cross-link was considered. A mechanism of formation of pyridinoline was postulated that, together with other evidence, appears to support the view that, in cartilage, pyridinoline acts primarily as an intramicrofibrillar cross-link and does not contribute to increased stability during maturation through lateral aggregation and bonding of filaments.

In the preceding paper (Robins, 1983), structural analysis of pyridinoline confirmed its existence as a 3-hydroxypyridinium compound. Evidence for the function of pyridinoline as an intermolecular cross-link was provided by analyses of Pronase (Fujimoto & Moriguchi, 1978) or thermolysin (Fujimoto, 1980) digests of bovine tendon collagen: pyridinoline-containing peptides were isolated and, although not fully characterized, their amino acid compositions were consistent with their proposed derivation from the C-terminal non-helical region and a helical portion at the 'overlap' region.

The present paper describes the isolation of pyridinoline-containing peptides from CNBr digests of bovine articular cartilage. This form of digestion was chosen to provide larger, more easily characterized, products than those from enzymic digests. Amino acid sequence studies of the isolated peptides indicated that pyridinoline is located at two sites in the molecule, involving both the N- and the C-terminal non-helical regions. The implications of these results on fibril organization during tissue maturation are considered.

Experimental

Materials

Phenyl isothiocyanate was purchased from Eastman-Kodak, Liverpool, U.K. Pyridine and ethyl acetate (AnalaR grade) were from BDH Chemicals, Poole, Dorset, U.K., and were redistilled before use. Trypsin (type III) and bacterial collagenase (type VII) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of the purest grade available.

Tissue preparation

Bovine articular cartilage from femoral heads of 18-month-old animals was dissected with a scalpel, frozen in liquid N₂ and powdered in a Moulinex grinder. The tissue was extracted with 4 M-guanidinium chloride at room temperature for 24 h to remove proteoglycans (Miller & Lunde, 1973), washed with water and freeze-dried.

Digestion with CNBr

The freeze-dried tissue (2g) was suspended in 70% (v/v) formic acid (400 ml) and flushed with N₂

for 20 min, and solid CNBr (10 g) was then added. Digestion was continued at 29°C for 5 h, after which time the material was diluted 10-fold with water and freeze-dried. The product was redissolved in water (300 ml), centrifuged at 40000g for 30 min and the supernatant solution was again freeze-dried. Analysis of acid hydrolysates of the supernatant and residue material indicated that CNBr digestion solubilized 97% of the hydroxyproline and 93% of the pyridinoline.

Ion-exchange chromatography

CM-cellulose chromatography was used for the initial separation of CNBr digests. The column (2.6 cm × 14 cm) was equilibrated at 42°C with a sodium citrate buffer, pH 3.4 (3.1 g of citric acid and 1.5 g of trisodium citrate dihydrate per litre) containing 20 mM-NaCl, and peptides were eluted with a linear NaCl gradient formed by an Ultrograd mixer (LKB Instruments, South Croydon, Surrey, U.K.). Before chromatography, samples were dissolved in the start buffer and were dialysed at 4°C for 16 h against the same buffer: this procedure was shown to result in the loss of less than 5% of the pyridinoline fluorescence through the dialysis membrane. Re-chromatography of some peptides on CM-cellulose was performed at 42°C on a 1.6 cm × 15 cm column with sodium citrate buffer, pH 3.7 (2.1 g of citric acid and 1.5 g of trisodium citrate dihydrate per litre): the peptides were eluted at 60 ml/h with a linear gradient of 0–100 mM-NaCl over 960 ml. Phosphocellulose chromatography of small peptides was performed with a 1.6 cm × 8 cm column at 42°C with 1 mM-sodium acetate buffer, pH 3.8 (Miller, 1972).

Gel filtration

Peptide fractions from CM-cellulose chromatography were separated on a column (2.6 cm × 85 cm) of Ultrogel AcA-44 run at 11 ml/h with 1 M-CaCl₂/50 mM-Tris/HCl buffer, pH 7.6, as eluent. Enzymically cleaved peptides were fractionated on a Bio-Gel P6 column (1.6 cm × 140 cm) eluted at 6 ml/h with 0.1 M-acetic acid. Peptide samples from the ion-exchange and Ultrogel columns were desalted on a 3.2 cm × 50 cm column of Bio-Gel P2 (100–200 mesh) eluted with 0.1 M-acetic acid.

Enzymic cleavage

Peptide PCP1 (7 mg) was dissolved in 0.1 M-Tris/HCl buffer, pH 7.6 (1.5 ml), containing 1 mM-CaCl₂. After the addition of trypsin (0.4 mg), digestion was continued at 37°C for 6 h or 16 h. One drop of acetic acid was then added and the complete digest was submitted to gel filtration. Digestions with collagenase were performed under similar conditions for 8 h.

Amino acid sequence analysis

Sequential Edman degradation of the cross-linked peptides (250–400 nmol) was performed manually essentially by the method described by Allen (1981). The amino acid phenylthiohydantoin derivatives were identified and quantified by high-pressure liquid chromatography on a μ Bondapak C₁₈ column (Waters Associates, Northwich, Cheshire, U.K.) with a methanol solvent system (Bhown *et al.*, 1978). Some residues were identified by using the dansyl chloride method (Gray, 1972), and a subtractive Edman degradation procedure (Balian *et al.*, 1971) was used for sequencing some small peptides.

Amino acid analysis

Peptides were hydrolysed in constant-boiling HCl *in vacuo* at 108°C for 24 h and were analysed on a Locarte amino acid analyser with a dual-column procedure. Pyridinoline was determined separately as described in the preceding paper (Robins, 1983).

Results

Isolation of pyridinoline-containing peptides

Initial fractionation on CM-cellulose of CNBr-cleavage peptides of 18-month-old bovine articular cartilage showed that the pyridinoline fluorescence was not randomly distributed but was present in specific peptides (Fig. 1). Pooled material, designated fraction 1 and fraction 2, was re-chromatographed on Ultrogel AcA-44 (Fig. 2). Fraction 2 revealed a fluorescent peak with an apparent M_r of 9200 that appeared to comprise a single peptide (PCP1), as judged by the presence of a single band by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and a single fluorescent peak on phosphocellulose chromatography under the conditions described in the legend to Fig. 3 (results not shown). The fluorescent material in fraction 1 had an apparent M_r of 11600, and this material was rechromatographed on CM-cellulose at pH 3.7 to give a single major fluorescent component, designated PCP2, that was well separated from other peptides.

Amino acid compositions of the pyridinoline-containing peptides are shown in Table 1. Peptide PCP1 corresponds closely in composition to the values for a peptide containing one CB12 and two CB14 chains calculated from published data (Butler *et al.*, 1976; Miller, 1972). Some differences in composition are, however, evident, particularly in the small amounts in peptide PCP1 of threonine, homoserine, isoleucine and tyrosine. The composition of peptide PCP2 was similar to that calculated for a peptide linking CB9,7 with two CB4 chains (Miller & Lunde, 1973), except for slight differences in the isoleucine/leucine ratio.

Fig. 1. *CM-cellulose chromatography of cartilage collagen peptides*

Approx. 250mg of CNBr-cleavage peptides of bovine articular cartilage were applied to the column, previously equilibrated with sodium citrate buffer, pH 3.4 (see the Experimental section for details). The arrow denotes the start of a linear gradient of 20–130mM-NaCl. —, A_{230} of the effluent; -----, relative fluorescence emission at 400nm with excitation at 295nm. The pooled fractions marked by the bars denoted 1 and 2 were separately desalted and rechromatographed (Fig. 2).

Fig. 2. *Gel filtration of CM-cellulose-derived peptide fractions*

Peptide fractions from CM-cellulose chromatography (Fig. 1) were chromatographed on Ultrogel AcA-44 as described in the Experimental section. —, A_{230} of the effluent; -----, relative fluorescence emission at 400nm with excitation at 325nm. (a) Elution profile of fraction 2 from the CM-cellulose chromatogram: the pooled fractions marked by the bar were designated peptide PCP1. (b) Elution profile of fraction 1 from the CM-cellulose chromatogram: fractions marked by the bar were re-chromatographed on CM-cellulose.

Sequence studies

Peptides PCP1 and PCP2 were subjected to sequential Edman degradations, and the yields of amino acid phenylthiohydantoin derivatives obtained at each cycle are shown in Table 2. Generally, two major peaks were detected at each stage, although some ambiguity was introduced at cycle 7 of peptide PCP1 and cycle 2 of peptide PCP2, for

which only glycine phenylthiohydantoin derivative was detected. For peptide PCP1, evidence from tryptic peptides (see below) confirmed that glycine occupied the *N*-terminal position of each chain at that stage.

After digestion of peptide PCP1 with trypsin for 6h, chromatography on Bio-Gel P6 separated a number of peptides, the fluorescent fractions of

Table 1. Amino acid compositions of CNBr-cleavage peptides from bovine articular cartilage

Compositions of peptides PCP1 and PCP2 are given as mol of residues/mol of peptide and are the mean values of three determinations. The compositions are compared with values for the sum of one CB12 and two CB14 chains [$\alpha 1(\text{II})\text{-CB}(12 + 14 + 14)$] calculated from the data of Butler *et al.* (1976) and Miller (1972) and with values for the sum of one CB9,7 chain and two CB4 chains [$\alpha 1(\text{II})\text{-CB}(9,7 + 4 + 4)$] calculated from Miller & Lunde (1973). Values of less than 0.2 are omitted (—)

	Composition (mol of residue/mol of peptide)			
	PCP1	CB(12 + 14 + 14)	PCP2	CB(9,7 + 4 + 4)
3-Hyp	—	—	1.8	2
4-Hyp	12	12	11	9
Asp	6.9	6	8.1	8
Thr	1.8	3	2.9	3
Ser	5.9	6	4.6	5
Hse*	1.6	3	2.6	3
Glu	12	10	12	12
Pro	12	10	19	20
Gly	33	34	43	46
Ala	8.9	9	11	12
Val	2.3	2	1.8	1
Ile	0.8	2	2.4	3
Leu	4.9	4	3.9	3
Tyr	1.7	3	—	—
Phe	2.8	3	2.7	3
Hyl	1.9	5 (2)†	1.4	4 (1)†
Lys	2.9	3	1.9	1
His	1.2	1	1.0	1
Arg	6.5	6	7.5	6
Pyridin-oline	0.7	—	0.8	—
Total residues	120	122	140	142

* Includes homoserine lactone.

† Numbers in parentheses account for involvement of three hydroxylysine residues in the formation of pyridinoline.

which were re-chromatographed on phosphocellulose (Fig. 3). The amino acid compositions of the isolated peptides are shown in Table 3. Although several of the peptides were impure and not fully characterized, their compositions confirmed the presence of the $\alpha 1(\text{II})\text{-CB}12$ chain. Thus, according to the residue numbering system of Butler *et al.* (1976), peptide T2 corresponds to the C-terminal portion, peptide T4 comprises residues 4–23, peptide T9 comprises residues 24–27 and peptide T3 constitutes residues 52–69 arising from partial cleavage at hydroxylysine-69. Longer treatment with trypsin (16 h) resulted in increased yields of peptide T3 with a concomitant decrease in the amount of peptide T2. Peptide T7 was sequenced by sub-

Table 2. Edman degradations of peptides PCP1 and PCP2

Amino acid phenylthiohydantoin derivatives from each degradation cycle were identified and quantified by high-pressure liquid chromatography (see the Experimental section). The yields (nmol) are expressed as a percentage of the starting material of peptides PCP1 (400 nmol) and PCP2 (250 nmol). In addition to those samples in which glycine phenylthiohydantoin derivative was a major component, small amounts of this derivative were detected at all other degradation cycles.

Degradation cycle	Amino acid phenylthiohydantoin derivative (% yield)	
	PCP1	PCP2
	Gly (25); Ser (8)*	Hyp (21); Ala (32)
1	Pro (28); Ala (66)	Gly (45)
2	Arg (40); Phe (55)	Pro (20); Gly (33)
3	Gly (28); Ala (30)	Gln/Glu (12); Phe (16)
4	Pro (28); Gly (50)	Gly (16); Asn/Asp (17)
5	Hyp (26); Leu (45)	Pro (10); Glu (32)
6	Gly (42)	Arg (10); Glu (10)
7	Pro (15); Gln/Glu (30)	Gly (16)
8	Hyp (20); Arg (28)	
9	Gly (20); Glu (28)	
10	Lys (5); Glu (12)	
11	Hyp (13); Gly (10)	
12	Gly (16)	
13	Asp (12)	
14		

* The presence of N-terminal serine was confirmed by dansylation.

tractive Edman degradation and was shown to comprise mainly residues 28–36 of the CB12 chain. Similar analysis of peptide T9 indicated that the major component had the sequence Ala-Gly-Leu-Gly-Glx-Arg, with a minor constituent, T9a, containing in addition the N-terminal sequence Ser-Ala-Phe-; again, the yield of peptide T9 was increased by longer treatment with trypsin. Peptides T5 and T6 had virtually identical amino acid compositions, despite their partial separation on phosphocellulose: slight differences in amidation may be responsible for this phenomenon. The data allow the partial sequence of peptide PCP1 to be deduced as shown in Fig. 4. The involvement of hydroxylysine-48 of the CB12 chain in the cross-link was indicated by the composition of the tryptic peptides, T5 and T6, which contained both histidine and threonine, amino acid residues unique to that portion of the CB12 chain (Butler *et al.*, 1976). Collagenase digestion of these tryptic peptides followed by separation of the fluorescent components by gel filtration and phosphocellulose chromatography provided confirmatory evidence for

Fig. 3. Separation of peptide-PCP1-derived tryptic peptides by gel filtration and phosphocellulose chromatography

—, A_{230} of the effluent; ----, relative fluorescence emission at 400 nm with excitation at 295 nm. (a) After treatment of peptide PCP1 with trypsin for 6 h, the resultant peptides were chromatographed on Bio-Gel P6 as described in the Experimental section: the peptide fractions that were freeze-dried before further analysis are indicated. The fluorescent fractions marked by the bar were submitted to ion-exchange chromatography. (b) Phosphocellulose chromatography of the fluorescent fractions from the Bio-Gel P6 column, after equilibration of the column at 42°C with 1 mM-sodium acetate buffer, pH 3.8. The peptides were eluted at 60 ml/h, and the arrow indicates the start of a linear gradient of 0–0.2 M-NaCl over 480 ml. The peptide fractions designated T3–T6 were desalted for further analysis.

Table 3. Amino acid compositions of tryptic peptides of PCP1

The compositions are given as mol of residue/mol of peptide, and the total residues were calculated from the sum of the nearest integer values. Abbreviation: Pyd, pyridinoline.

	Composition (mol of residue/mol of peptide)							
	T2	T3	T4	T5	T6	T7	T8	T9
4-Hyp	3.4	1.9	3.3	2.9	2.8	1.4	—	—
Asp	2.4	1.0	2.1	1.8	1.5	0.2	—	—
Thr	0.3	—	—	1.0	1.0	—	—	—
Ser	3.2	—	—	0.4	0.5	0.2	0.3	0.9
Hse*	0.9	—	—	—	—	—	—	—
Glu	2.9	1.2	1.3	3.5	3.6	1.2	1.1	1.1
Pro	1.7	0.2	2.8	2.8	2.7	1.9	—	—
Gly	12.3	6.4	7.2	7.4	7.3	3.6	2.4	1.2
Ala	3.2	2.8	1.0	0.3	0.2	1.2	1.3	—
Val	1.0	0.8	—	0.8	0.9	—	—	—
Leu	1.1	1.0	0.3	2.1	1.9	—	1.0	—
Tyr	0.9	0.9	—	0.9	0.8	—	—	—
Phe	0.2	—	0.2	1.1	1.1	—	0.2	—
Hyl	1.5	1.8	—	—	—	—	—	—
Lys	0.4	0.2	2.8	—	—	—	—	—
His	—	—	—	0.9	1.0	—	—	—
Arg	—	—	—	1.0	0.9	1.0	1.0	1.0
Pyd	—	—	—	0.8	1.0	—	—	—
Total residues	34	18	20	28	28	10	6	4

* Includes homoserine lactone.

the amino acid composition of the C-terminal portion of the CB14 peptide.

Discussion

The results for peptide PCP1 show conclusively that, in cartilage collagen, pyridinoline links two C-terminal telopeptide chains (CB14) with the helical peptide, CB12. In addition to the presence of a stoichiometric amount of the trifunctional cross-link (Table 1), the relative yields of amino acid phenylthiohydantoin derivatives in the early cycles of the Edman degradation (Table 2) provide further evidence for the presence of three chains in the peptide. In characterizing this material, it would clearly have been preferable to effect a selective cleavage at the

cross-link and to identify the individual chains released. Pyridinoline is susceptible to the action of u.v. light (Robins, 1982; Sakura *et al.*, 1982), but irradiation of peptide PCP1 was not successful in cleaving the peptide, although the pyridinoline itself was destroyed (S. P. Robins, unpublished work). The fact that the sequence of peptide $\alpha 1(\text{II})$ -CB12 from bovine cartilage was known (Butler *et al.*, 1976) made feasible analysis of the cross-linked peptide by direct sequencing techniques. As shown in Fig. 4, the partial sequence obtained of the C-terminal peptide (CB14) differed in some respects from that determined from chick complementary DNA (Miller, 1983).

The suggested sequence around the cross-link in the non-helical chains (Fig. 4) was derived mainly from the composition of the tryptic peptides T5 and T6 (Table 3) by subtracting residues 37–51 of peptide CB12 (Butler *et al.*, 1976), the tryptic fragment involved in cross-linking. This resulted in a composition for residues 10^c to the C-terminus of each chain: Asp (1.0), Ser (0.2), Glu (1.8), Pro (1.0), Gly (1.2), Leu (0.5), Tyr (0.5). Similar results were obtained from the composition of a collagenase-treated tryptic peptide, except for somewhat lower values of leucine and tyrosine. The reason for

The sequence was obtained by sequential Edman degradation and by analysis of tryptic peptides. The arrows indicate full cleavage sites by trypsin and the broken arrow a partial cleavage site owing to the presence of chymotryptic activity in the enzyme preparation: these gave rise to peptides T9 and T9a from the CB14 chain (see the text for details). The results of 16 cycles of the Edman degradation were consistent with the known sequence of the CB12 chain (Butler *et al.*, 1976), and this was used to infer the sequence of the CB14 chains. The portions of the latter in parentheses are likely sequences based on amino acid compositional data. The linkage site of pyridinolone (Pyd) is indicated. The sequence of the CB14 chain from chick cartilage is shown for comparison (Miller, 1983).

non-stoichiometric amounts of serine, leucine and tyrosine residues cannot be fully explained, but may be indicative of non-specific proteolysis of the non-helical portions of the peptide towards their C-terminal end. The lower than expected yield of homoserine in peptide PCP1 (Table 1) is consistent with this interpretation. This form of degradation had also been noted previously during the identification of peptide CB14 (Miller, 1972) and in attempts to isolate the bifunctional cross-linked peptide CB12 $\times$ 14 (E. J. Miller, personal communication). The possibility should be borne in mind that some of the observed anomalies may result from true sequence heterogeneity (Butler *et al.*, 1977). Also, as cartilage contains a small proportion of genetically distinct collagens (Burgeson & Hollister, 1979; Shimokomaki *et al.*, 1980; Ayad *et al.*, 1981), peptide heterogeneity through formation of cross-links with these minor collagens should perhaps be considered in view of the known occurrence of covalent bonding between type I and type III collagen in human leiomyoma and calf aorta (Henkel & Glanville, 1982).

Although peptide PCP2 has not yet been fully characterized, its amino acid composition (Table 1) suggests that it contains two *N*-terminal non-helical peptides (CB4) linked by pyridinoline to an $\alpha 1(\text{II})$ -CB9,7 chain in the helical portion of the molecule. This peptide contained only hydroxyproline and alanine as *N*-terminal residues. From the direct sequence analysis (Table 2), some preliminary enzymic degradation studies (not shown) and the amino acid compositional data for the $\alpha 1(\text{II})$ -CB4

chain given by Miller & Lunde (1973), the probable sequence of the *N*-terminal region of the CB9,7 chain was Hyp-Gly-Pro-Gln-Gly-Pro-Arg-Gly- and that of the CB4 chain was Ala-Gly-Gly-Phe-Asn-Glu(Glu,Gly,Pvd,Ala,Ala,Gly,Hse).

It can be concluded, therefore, that pyridinoline functions as a cross-link in cartilage collagen by linking both the *N*- and the *C*-terminal ends of the molecule to points on the helix of neighbouring molecules such that the 4D stagger (Hodge & Petruska, 1963) is stabilized (Fig. 5). In 18-month-old cartilage, the isolated peptides accounted for about 40% of the total pyridinoline fluorescence, and a number of other partially purified peptides containing pyridinoline appeared to be derived from the same location, but contained additional peptides owing to incomplete cleavage at some methionine residues.

Mechanism of pyridinoline formation

A knowledge of the mechanism of formation of pyridinoline has important implications in understanding the microfibrillar organization of collagen. From the structure of pyridinoline, it was proposed that biosynthesis of the molecule occurred through the reaction of two hydroxyallysine residues and one hydroxylysine residue (Fujimoto *et al.*, 1978). However, Eyre (1980) suggested that interaction of two bifunctional cross-links, hydroxylysino-5-oxonorleucine, was the most likely mechanism of formation. This would necessarily involve reaction between microfilaments in register, which is accommodated by most current models of molecular pack-

ing (Bailey *et al.*, 1980; Piez & Trus, 1981). An alternative mechanism summarized in Scheme 1 involves an 'aldol' condensation of hydroxyallysine with the bifunctional cross-link. This mechanism imposes less constraints on the arrangements of microfilaments, as formation of pyridinoline could also occur through an intramolecular reaction of hydroxylysino-5-oxonorleucine (Fig. 5); possible steric hindrance to the approach of two glycosylated bifunctional cross-links would also be ob-

viated. Clearly, the mechanism proposed implies that pyridinoline in cartilage may act as a bifunctional cross-link rather than a trifunctional intermicrofibrillar bond. As reactive aldehyde groups may not be expected to remain available for reaction within the fibril for extended periods, it is likely that, in cartilage, further reaction of hydroxyallysine with the reducible cross-link is relatively rapid: in support of this interpretation, there is very little increase with age in the pyridinoline content of cartilage (Eyre &

Fig. 5. Possible locations of pyridinoline cross-links in type II collagen

The diagram represents triple-chain molecules in quarter-staggered array (Hodge & Petruska, 1963) within each microfibril. The arrow indicates the interface between two microfibrils aligned in register such that their *N*- and *C*-terminal ends are adjacent. The order of CNBr-cleavage peptides is shown according to the nomenclature of Miller & Lunde (1973). The heavy lines indicate the possible locations of the cross-linked peptides isolated in the present study, those on the right of the diagram corresponding to intra-microfibrillar bonding and those on the left depicting inter-microfibrillar bonds.

Scheme 1. Proposed mechanism of formation of pyridinoline

Reaction of hydroxylysino-5-oxonorleucine with the keto form of hydroxyallysine is proposed to occur through an 'aldol'-type condensation. This is followed by an internal condensation reaction resulting in the formation of dihydropyridinoline, which would then undergo spontaneous oxidation, analogous to the reaction proposed for the formation of desmosines (Davis & Anwar, 1970).

Oguchi, 1980; Robins, 1983). In other tissues, such as bone and tendon, there appears to be a gradual increase in pyridinoline content with age, and a mechanism involving intermicrofibrillar bonding may be more prevalent. However, pyridinoline is absent from 'poly- α ICB6', a polymer, isolated from tendon, apparently from lateral aggregation of microfibrils (Light & Bailey, 1980*a,b*). By contrast, pyridinoline is present in the collagen of sea-cucumber tubules (Eyre & Oguchi, 1980), a tissue in which the characteristically low tensile strength may be due to the lack of transverse bonding (Bailey *et al.*, 1982).

It may be concluded, therefore, that, although pyridinoline constitutes a major intermolecular cross-link in several tissues and its formation probably accounts for the disappearance during maturation of the keto-amine reducible cross-links, there is as yet no evidence that pyridinoline contributes to the increased stability of mature collagens through its multifunctional cross-linking capability.

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