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An Investigation of Pyridinoline, a Putative Collagen Cross-Link

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A component, termed pyridinoline, has been reported to be derived from 'lysine aldehyde' (2,6-diaminohexanaldehyde) and designated as the stable cross-link of mature collagen. Commercially prepared collagen and freshly obtained mature bovine tendon collagen were both investigated with regard to their pyridinoline content. Both sources of material could be depleted of this component by mild washing procedures. Pepsin-solubilized collagen and peptides derived from CNBr cleavage of intact collagen did not contain the compound. Pure pyridinoline was isolated and shown to be hydrolysed by water, as previously reported, but neither hydroxylysine nor lysine could be detected in the hyrolysate. The results clearly demonstrate that pyridinoline is not a cross-linking component of collagen.

The problem of identity of the stable cross-link of collagen from mature tissues has defied considerable effort by many workers. In recent years, two compounds have been isolated from insoluble (mature) collagen and their structures determined. Housley *et al.* (1975) isolated and partially characterized 'hydroxy-aldol histidine', but no further evidence for the existence of this compound *in vivo* has been forthcoming, and its structure has not been independently reported. Another compound, isolated by Fujimoto *et al.* (1977) and named pyridinoline, has received considerable attention since its discovery, and Fujimoto and his co-workers have demonstrated the accumulation of this component in collagenous connective tissues during aging and its association with a small, apparently cross-linked, peptide (Moriguchi & Fujimoto, 1978; Fujimoto & Moriguchi, 1978). The structure of the compound has been based on ^{1}H n.m.r. and ^{13}C n.m.r. spectroscopy, but no mass spectrum has been published. The compound was reported to be hydrolysed by water at 110°C but was stable in 6M-HCl under the same conditions. Degradation products of pyridinoline hydrolysed in water were shown to include hyroxylysine by paper chromatography. From this evidence the compound was suggested to be a derivative of a pyridine ring formed by the combination of two 'hydroxylysinaldehyde' residues and one hydroxylysine residue (Fujimoto *et al.*, 1978). This proposed structure has not been confirmed by synthesis.

Here we report evidence that conclusively shows that pyridinoline is not a collagen cross-link and is not, in fact, even associated with collagen. We have prepared the compound and have confirmed its degradation by hydrolysis in water. However, we could not confirm the presence of either lysine or hydroxylysine in the hydrolysate. We suggest that pyridinoline is an artefact produced from collagen and contaminating proteins during acid hydrolysis.

Materials and Methods

Preparation of collagen

Collagen was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. or as bovine Achilles tendons from freshly slaughtered animals. Commercially obtained collagen was investigated as supplied, as well as after washing with either acid/urea buffer (4M-urea in 0.5M-acetic acid) or water. Bovine tendon collagen was also investigated in the unwashed state, or after washing with acid/urea buffer or with neutral salt solution (0.15M-NaCl containing 0.02M-sodium phosphate, pH 7.4).

Both crude bovine tendon and commercial collagen were washed with acid/urea buffer either at 20°C for 24h or at 60°C for 8h. Water and neutral-salt-solution washes were always carried out at 20°C for several days. Urea-washed collagen

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was briefly rinsed with water on a glass sinter to remove urea. After washing procedures the collagen samples were freeze-dried.

Pepsin solubilization of bovine tendon collagen

Unwashed bovine tendon from mature animals was finely chopped and suspended in 0.5 M-acetic acid by use of a Polytron homogenizer at full speed for 10–15 s at 4°C. Pepsin (Worthington) was added to give a final enzyme/substrate ratio of 1:10 and the suspension was incubated at 4°C for 24 h. Insoluble material was then collected by centrifugation at 40000 *g* for 30 min and immediately freeze-dried. Soluble collagen was precipitated from the supernatant with 0.9 M-NaCl and was collected by centrifugation at 40000 *g* for 30 min. The supernatant was concentrated by rotary evaporation and was desalted into water on Sephadex G-10 (Pharmacia, Uppsala, Sweden). Both dialysed soluble collagen and the desalted supernatant were freeze-dried.

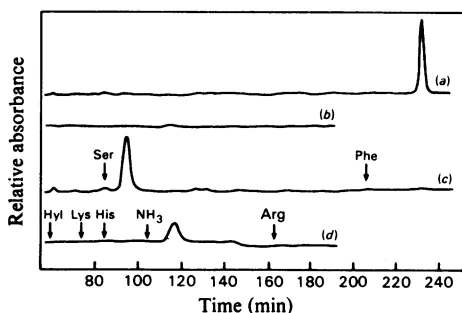

Fig. 1. *Amino acid analysis*
Purified pyridinoline and pyridinoline derivatives were subjected to ion-exchange chromatography in standard citrate-buffer systems on a Jeol amino acid analyser. (*a*) Purified pyridinoline (see the Materials and Methods section) plus an equal proportion of pyridinoline kindly supplied by Dr. D. Fujimoto after resolution of acid and neutral components. (*b*) As (*a*) after resolution of basic components. (*c*) Pyridinoline degradation products from a 24 h hydrolysis in water at 110°C after resolution of acid and neutral components. (*d*) As (*c*) after resolution of basic components. Mobilities of standard amino acids are indicated. Abbreviation used: Hyl, hydroxylysine.

Preparation of peptides derived from CNBr cleavage of collagen ('CNBr peptides')

Unwashed bovine achilles tendon from mature animals was digested with CNBr as previously described (Light & Bailey, 1979*a*). The CNBr peptides were then resolved by gel filtration on A1.5M agarose (Bio-Rad, Bromley, Kent, U.K.) in 1 M-$CaCl_2$ containing 0.05 M-Tris/HCl, pH 7.5, as described by Light & Bailey (1979*b*). All peptide material, as monitored at 236 nm, was pooled in seven fractions, exhaustively dialysed (or desalted on Sephadex G-10 in the case of dialysable peptides) and freeze-dried.

Preparation of pyridinoline and amino acid-assay procedure

Pyridinoline was prepared in bulk from an acid hydrolysate of decalcified mature bovine bone by the method described by Fujimoto *et al.* (1978). The pure material was shown to co-chromatograph on the Jeol amino acid analyser with a sample of pyridinoline kindly supplied by Dr. D. Fujimoto. The compound had fluorescence and u.v.-absorption spectra (Fig. 2) identical with those previously published for pyridinoline (Fujimoto *et al.*, 1977).

Washed and unwashed samples of collagen and the various fractions from pepsin-treated bovine tendon as well as partially purified CNBr peptides were hydrolysed in 6 M-HCl under vacuum for 24 h at 110°C at a final concentration of 1–2 mg/ml. The samples were dried *in vacuo* and pyridinoline content was assayed by amino acid analysis. Pyridinoline was eluted after phenylalanine and before

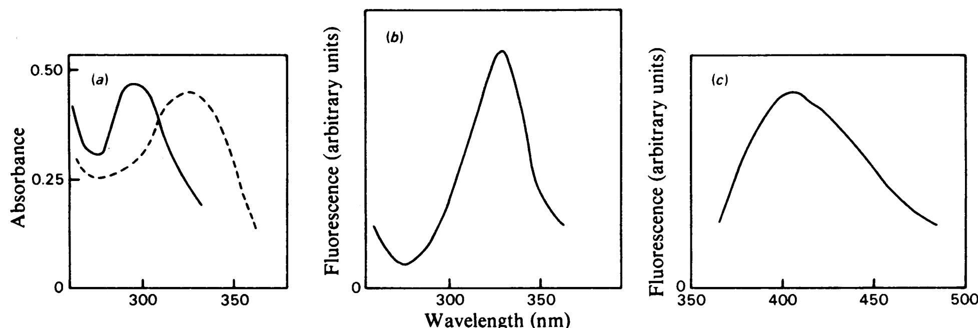

Fig. 2. *U.v.-absorption and fluorescence-activation and -emission spectra of purified pyridinoline*
(*a*) U.v.-absorption spectrum in 0.1 M-HCl (——) and in 0.1 M-NaOH (----). (*b*) Activation spectrum in 0.02 M-sodium phosphate buffer, pH 7.4; emission was monitored at 400 nm. (*c*) Fluorescence spectrum in 0.02 M-sodium phosphate buffer, pH 7.4; excitation was at 325 nm.

N-acetylglucosamine. As the proportion of pyridinoline was small (0.01–0.05% of the total material), 10mg of hydrolysate was loaded for a standard run on the amino acid analyser, but the flow of ninhydrin was switched on late so that only phenylalanine and pyridinoline peaks were recorded. Pyridinoline was calculated with respect to total material analysed and to phenylalanine content.

Pure pyridinoline was incubated in sealed tubes at 110°C for 24h in both distilled, de-ionized water and 6M-HCl. The solutions were dried *in vacuo* and the samples were analysed for amino acid content on the Jeol amino acid analyser.

Results

Table 1 shows the pyridinoline content of untreated Worthington collagen and bovine tendon collagen from immature (6 months) and mature (10 years) animals. The tendons of mature animals contain the highest amounts of the compound, with the commercial collagen containing 50% of this amount; the tendons of immature animals contain 24% of that in the tendons of mature animals. However, the amounts of pyridinoline in both the Worthington and mature-animal tendon collagen could be significantly decreased by washing in 4M-urea containing 0.5M-acetic acid at 20°C (decreased by 69 and 50% respectively). The amount of the component in mature-animal tendon was decreased by 53% by extensive washing in neutral-salt solution and by 44% in commercially obtained collagen by extensive washing in water. In each case the washings (after desalting, if necessary) contained no significant proportions of pyridinoline. After washing with acid/urea buffer for 8h at higher temperatures, neither mature-animal tendon nor Worthington collagen contained detectable amounts of pyridinoline. Both re-precipitated pepsin-solubilized collagen from mature-animal tendon and the insoluble residue contained 90–95% less pyridinoline than the starting sample. Further, only trace amounts were observed in the supernatant fraction. A similar result was obtained when Worthington collagen was used as the starting material. Partially purified CNBr peptides of mature-animal tendon contained no detectable pyridinoline.

Figs. 1(*a*) and 1(*b*) show elution profiles for purified pyridinoline on ion-exchange chromatography using the Jeol amino acid analyser. Fig. 1(*c*) and 1(*d*) show a similar profile of pyridinoline after hydrolysis in water at 110°C. Two unidentified ninhydrin-positive components were observed. No hydroxylysine or lysine was present in the hydrolysate. Pyridinoline was found to be stable in 6M-HCl at 110°C for 24h.

Discussion

We have shown that both commercially obtained collagen and crude bovine tendon collagen contained the putative cross-link compound pyridinoline. Immature bovine tendon contained only 24% of the amount of the compound found in mature tendon, apparently confirming the previous results of Moriguchi & Fujimoto (1978) showing that it accumulates during aging. However, the possibility that pyridinoline is actually derived from collagen is thrown into considerable doubt by the results shown in Table 1.

Table 1. *Pyridinoline content of treated and untreated collagen fractions*

Pyridinoline was quantified by its ninhydrin response relative to the total amount of collagen analysed. Abbreviation used: nd, not detectable.

Sample	Treatment	Pyridinoline (arbitrary units)	Pyridinoline remaining after treatment (%)
Bovine tendon from mature (10-year-old) animals	None	13.6	100
	Acid/urea-washed at 20°C	6.9	51
	Acid/urea-washed at 60°C	nd	0
	NaCl-washed at 20°C	6.4	47
	Pepsin-solubilized collagen	0.8	6
	Pepsin-insoluble collagen	0.4	3
	Supernatant from pepsin solubilization	0.2	1.5
	CNBr peptides resolved by gel filtration	nd	0
Bovine tendon from immature (6-month-old) animals	None	3.3	100
	NaCl-washed at 20°C	1.6	48
Worthington collagen	None	6.8	100
	Water-washed at 20°C	4.5	66
	Water-soluble fraction	0.3	4
	Acid/urea-washed at 20°C	2.1	31
	Acid/urea-washed at 60°C	nd	0

The reprecipitated soluble fraction obtained after pepsin treatment of both mature-animal tendon and Worthington collagen contained only 5–10% of the amount of pyridinoline observed in the original collagen. The reducible cross-links of immature collagens form between the non-helical ends of the molecule and helical regions of other adjacent molecules and have been demonstrated by the isolation of two cross-linked peptides (Miller & Robertson, 1973; Henkel *et al.*, 1976). On the reasonable assumption that these stable cross-links are derived from the intermediate reducible bonds that are known to be located between the non-helical regions and specific triple-helical regions, the cross-link would remain associated with the same helical regions after pepsin cleavage of the non-helical ends of the molecule at 4°C. The absence of pyridinoline from the pepsin-solubilized collagen demonstrates that it cannot be derived from the reducible cross-links. It could be argued, however, that it is a new cross-link formed exclusively in the pepsin-sensitive non-helical regions. However, this is not borne out by investigation of the dialysable material obtained after pepsin degradation, which only contained trace amounts of pyridinoline.

The most crucial evidence against pyridinoline being a genuine collagen component is the decrease in the amount of the component after mild-washing procedures and also its absence from the washings obtained. Washing with various solutions at room temperature decreased the pyridinoline content of collagen by up to 69%, even though the solubility of the collagen was unaffected and was therefore still highly cross-linked by stable bonds. Washing in urea at 60°C completely removed all trace of the component. Although it could be argued that pyridinoline may be heat-labile under such conditions, the likelihood that such a compound would be present as a stable collagen cross-link is negligible when the cross-links of mature collagen are known to be extremely thermally stable. Attempts to identify pyridinoline in partially purified collagen CNBr peptides failed to detect any significant amounts.

The weight of evidence shows conclusively that pyridinoline is not a cross-link component derived from collagen but rather an artefact produced by the interaction of collagen and non-collagenous contaminants during acid hydrolysis. Finally, the proposed structure for this compound may well be incorrect, as water hydrolysis yielded no hydroxylysine or lysine as previously suggested (Fujimoto *et al.*, 1978).

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