Biochem. J. (1983) **215**, 167–173
Printed in Great Britain

Cross-linking of collagen

Isolation, structural characterization and glycosylation of pyridinoline

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(*Received 25 April 1983/Accepted 27 June 1983*)

A method for the isolation and purification of pyridinoline from bone collagen was developed, with the use of sulphonated polystyrene resins. The analytical techniques were used to quantify pyridinoline, for which hydroxyallysine is a known precursor, in a wide range of tissues. The structure of pyridinoline proposed by Fujimoto, Moriguchi, Ishida & Hayashi [(1978) *Biochem. Biophys. Res. Commun.* **84**, 52–57] was confirmed by ^{13}C-n.m.r. spectroscopy and fast-atom-bombardment mass spectrometry. At concentrations greater than about 0.1 mM, pyridinoline exhibited altered fluorescence properties that were consistent with excimer formation. From alkali hydrolysates of several different tissues, a fluorescent compound was purified by gel filtration and ion-exchange chromatography and was shown to be galactosylpyridinoline. This derivative was very labile to acid treatment compared with the bifunctional cross-link analogues, and was completely converted into free pyridinoline by heating at 108°C for 8h in 0.1 M-HCl. Galactosylpyridinoline was also partially converted into free pyridinoline by prolonged alkali hydrolysis. This lability, which could also apply to other multifunctional cross-link derivatives, may explain the fact that no disaccharide derivatives of pyridinoline were isolated.

The lysyl oxidase-mediated cross-linking system of collagen leads to the formation of reducible intermediates that appear to be converted into stable non-reducible compounds during tissue maturation (Bailey *et al.*, 1974). A fluorescent compound, termed pyridinoline, was isolated from bovine Achilles tendon or bone and was characterized by u.v. and n.m.r. spectroscopy as a 3-hydroxypyridinium compound (Fujimoto *et al.*, 1977, 1978). This material was proposed as a non-reducible trifunctional collagen cross-link of mature collagen (Fujimoto & Moriguchi, 1978).

The proposed structure and function of pyridinoline were both questioned by Elsden *et al.* (1980) as a result of experiments in which pyridinoline was apparently removed from bovine tendon by mild washing procedures; also, after hydrolysis of isolated pyridinoline in water, no lysine or hydroxylysine was detected (Elsden *et al.*, 1980). These results were disputed by Fujimoto (1980), and subsequently a number of other groups have reported indirect experiments supporting the existence of pyridinoline in various forms of extracted collagen and collagen peptides (Eyre & Oguchi, 1980; Tsuchikura *et al.*, 1981; Tsuda *et al.*, 1982).

The present paper describes the structural analysis of pyridinoline by ^{13}C-n.m.r. and fast-atom-bombardment mass spectrometry together with analysis of its fluorescence properties. Also, evidence for the presence of unusually labile glycosylated forms of the cross-link is presented.

Experimental and results

Materials

The diaphysial regions of long bones from 6–10-year-old sheep were frozen at −20°C, fragmented with a mallet and the marrow was removed. The frozen bone was then powdered in a hammer mill (Christy and Norris, Chelmsford, Essex, U.K.) and decalcified by two treatments with 10 vol. of 2 M-HCl, each for 30 min at room temperature. The material was washed extensively with cold water, during which most of the remaining fat was decanted off, and the decalcified bone was freeze-dried.

Isolation of pyridinoline

Powdered decalcified bone (125 g) was hydrolysed in 6 M-HCl (3 litres) under reflux with an N_2 bleed for 22 h. The cooled hydrolysate was filtered,

evaporated to dryness and dissolved in a solution containing trisodium citrate dihydrate (138g), which was then titrated to pH2.3 with 6M-HCl (final volume 7 litres). This solution was pumped on to a jacketed column (4cm × 45cm) of Duolite 225 (Na^+ form) at 400ml/h at room temperature, and was eluted with 67mM-sodium citrate, pH4.15, until most of the phenylalanine had been eluted (determined by monitoring column effluent at 257nm). This required about 5 litres of buffer that had previously been degassed under vacuum in preparation for raising the column temperature to 60°C for the elution of a pyridinoline fraction with the same buffer adjusted to pH5.0 by the addition of 20ml of 2M-NaOH/litre. Column effluent was monitored at 325nm, and the pyridinoline-containing fraction that was eluted between 900 and 1300ml was adjusted to pH2.3 with HCl. This fraction was re-chromatographed on a column (2.6cm × 80cm) of Duolite 225 equilibrated at 60°C with 67mM-sodium citrate buffer, pH4.25, and was eluted at 240ml/h with the same buffer. Pyridinoline, detected by monitoring the effluent at 295nm, was eluted between 1300 and 1750ml; this pooled fraction was adjusted to pH2.3 with HCl, and the volume was decreased by adsorption on a column (1.6cm × 30cm) of Duolite 225 equilibrated with 67mM-sodium citrate buffer, pH4.25, at 60°C followed by elution with 67mM-sodium citrate buffer, pH5.3. The pyridinoline fraction (about 80ml) was again adjusted to pH2.3 with HCl and submitted to a final purification step at pH4.25 on the extended basic column (0.9cm × 15cm) of a Locarte analyser under conditions described previously (Robins, 1982*a*). The detergent, Brij 35, was omitted from the buffers to facilitate concentration of the pooled pyridinoline fractions by evaporation *in vacuo* at 40°C. Finally, this solution was desalted by chromatography on a column (1.6cm × 90cm) of Bio-Gel P2 (200–400 mesh) with 0.1M-acetic acid as eluent. The yield of pyridinoline (acetate salt) from 125g of freeze-dried bone was 28.5mg.

Chromatographic analysis of pyridinoline

On chromatography at pH4.25 on an extended basic column of the amino acid analyser, pyridinoline was eluted as a partially resolved triple peak owing to the presence of diastereoisomers (see Fig. 6). For rapid quantitative analyses, chromatography with 67mM-sodium citrate buffer, pH4.25, on the 0.9cm × 6cm column of the analyser resulted in elution of pyridinoline as a single symmetrical peak (Fig. 1). This method was applicable directly for peptide hydrolysates, but for tissue samples a preliminary fractionation step on an extended basic column (0.9cm × 15cm) with pH4.70 buffer (Robins, 1982*a*) was necessary, followed by analysis of the appropriate fractions detected by fluorescence measurements. The results for quantitative pyridinoline analysis in various tissues are shown in Table 1. These values are based on a measured molar colour yield with ninhydrin for pyridinoline of 2.1 times that of lysine. The results support the

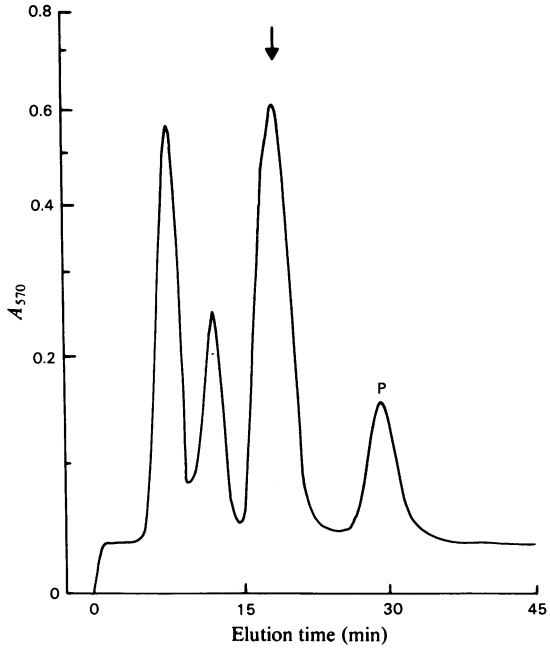

Fig. 1. *Chromatography of pyridinoline*
After fractionation of a tissue hydrolysate with pH4.70 sodium citrate buffer, chromatography at pH4.25 of the relevant fluorescent fractions resulted in a symmetrical peak for pyridinoline (P) by monitoring with ninhydrin (see the text for experimental details). The arrow indicates the elution position of tyrosine and phenylalanine.

Table 1. *Quantitative tissue distribution of pyridinoline*
Pyridinoline was determined with an amino acid analyser as described in the text. Hydroxyproline was measured separately in samples of each hydrolysate, and the results were calculated assuming 285mol of hydroxyproline/mol of collagen. Abbreviation: N.D., not determined.

Tissue	Age (years)	Pyridinoline (mol/mol of collagen)
Bovine articular cartilage	1.5	0.8
Bovine articular cartilage	10	0.7
Sheep bone (femur)	11	0.5
Bovine Achilles tendon	10	1.1
Rabbit intramuscular collagen	1.5	0.2
Human aorta (intima + media)	76	1.1
Bovine skin	10	N.D.

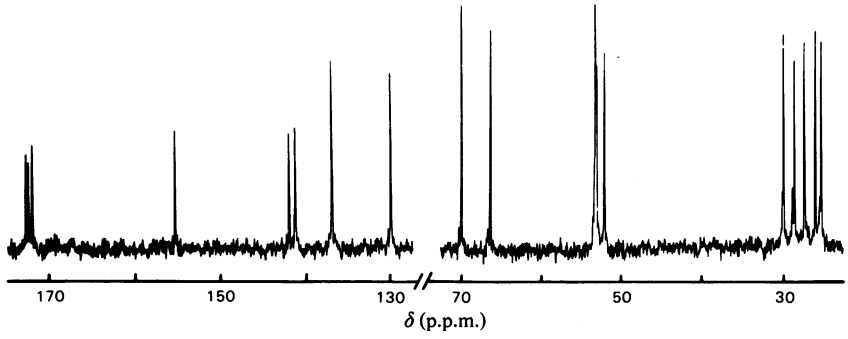

Fig. 2. *^{13}C-n.m.r. spectrum of pyridinoline*
The spectrum was recorded on a Brucker CXP 300 instrument with proton noise decoupled. Chemical shifts (δ) are given in p.p.m. downfield from the resonance of an external tetramethylsilane reference. No signals were recorded between 73 and 127 p.p.m., and this part of the spectrum is omitted (for assignments see Table 2).

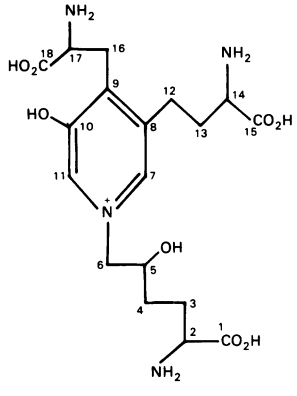

Fig. 3. *Proposed structure of pyridinoline*
The proposed structure has an M_r value of 429 corresponding to the molecular ion in the mass spectrum (Fig. 4): the C numbers refer to assignments of the ^{13}C chemical shifts (Table 2).

Table 2. *^{13}C-n.m.r. chemical shifts of pyridinoline*
Shifts at pH 2 and pH 7 are shown together with tentative assignments of the signals according to the numbering given in Fig. 3.

δ (p.p.m.)			Tentative assignment
pH 2	pH 7	Difference	
172.6	173.5	0.9	C-1
172.3	173.1	0.8	C-15
171.9	172.6	0.7	C-18
155.1	164.1	9.0	C-10
141.5	141.9	0.4	C-8
140.7	138.8	−1.9	C-9
136.4	131.0	−5.4	C-11
129.4	128.8	−0.6	C-7
69.6	69.1	−0.5	C-6
66.0	64.7	−1.3	C-5
53.2	53.7	0.5	C-2
53.0	53.7	0.7	C-14
52.1	53.7	1.6	C-17
30.2	30.3	0.1	C-3
28.8	28.3	−0.5	C-4
27.6	27.5	−0.1	C-12
26.3	26.0	−0.3	C-13
25.5	25.2	−0.3	C-16

conclusion that hydroxyallysine is necesary for pyridinoline formation, since all of the tissues examined except skin contain hydroxylysine residues in their non-helical peptides.

Analysis by ^{13}C n.m.r.

The acetate salt of pyridinoline was converted into the hydrochloride by dissolving the sample in 1 M-HCl and removing the acid by evaporation *in vacuo* at 40°C: the sample was re-dissolved in water and freeze-dried. For ^{13}C-n.m.r. spectrometry, the pyridinoline sample (45 mg) was dissolved in ^2H_2O (6 ml) to give a solution at pH 2, and the spectrum shown in Fig. 2 was recorded, in which distinct signals for each of 18 C atoms in the proposed structure (Fig. 3) are evident. The assignments shown in Table 2 are based on values for reference compounds (Horsley *et al.*, 1970; Morishima *et al.*, 1973) and were augmented by repeating the spectrum at pH 6.8. The differences shown in Table 2 were most marked in the signals due to C-10, C-9 and C-11, consistent with the effects of protonation of the ring hydroxy groups: also, in neutral solution the signals due to the α-carbon atoms were coincident.

Fast-atom-bombardment mass spectrometry

A solution of unmodified pyridinoline in 50 mM-HCl was applied directly to the glycerol-coated

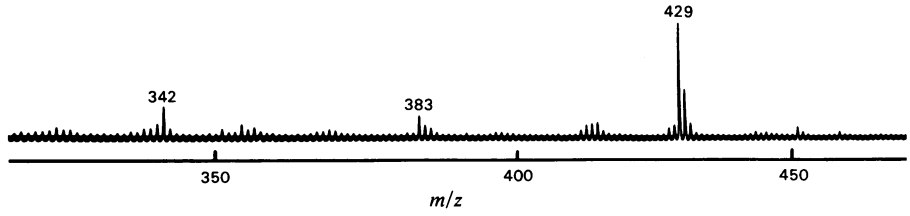

Fig. 4. *Partial fast-atom-bombardment mass spectrum of pyridinoline*
The positive ion spectrum was recorded on an MS 9 instrument fitted with a fast-atom-bombardment ionization source (Kratos, Manchester, U.K.).

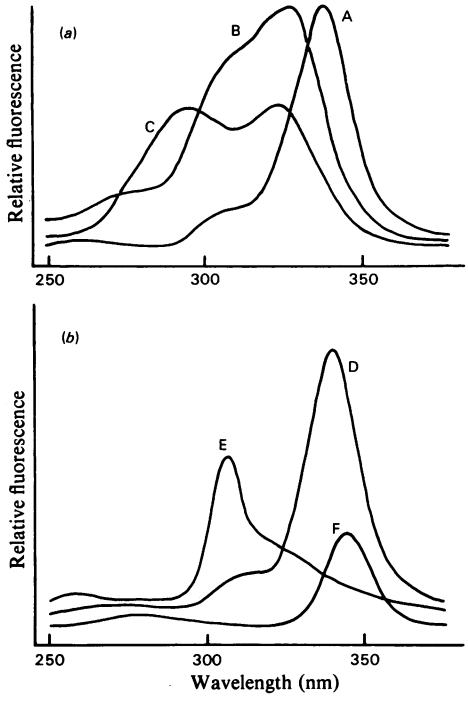

Fig. 5. *Fluorescence spectra of pyridinoline*
The uncorrected excitation spectra were recorded with emission at 400nm. (*a*) Spectra in solutions of 67mM-sodium citrate buffer, pH4.25, with pyridinoline concentrations of 1.6mM (A), 0.4mM (B) and 0.1mM (C); (*b*) spectra of 1.6mM-pyridinoline in solutions at pH4 (D), pH2 (E) and pH11 (F).

probe. The positive ion spectrum (Fig. 4) showed a prominent ion at *m/z* 429 corresponding to M^+, the pyridinium ion. As is characteristic for this type of spectrum (Barber *et al.*, 1982), little fragmentation occurred, although signals were present at *m/z* 383, 342 and 284 resulting from losses of a carboxy group, $-CH_2-CH(NH_2)-CO_2H$ and $-CH(OH)-[CH_2]_3-CH(NH_2)-CO_2H$ respectively.

Fluorescence properties

The fluorescence characteristics in dilute solution of pyridinoline isolated by the methods described in the present paper were identical with those described by Fujimoto *et al.* (1977). Thus an excitation maximum at 295nm (emission 400nm) in acid solution shifted at about pH4.2 to an excitation maximum at 325nm (emission 400nm) at neutral and alkaline pH (Robins, 1982*a*). At concentrations above about 0.1mM, pyridinoline exhibited alterations in its fluorescence spectra, as shown in Fig. 5: a 1.6mM-solution of pyridinoline showed an excitation maximum at 340nm (emission 400nm) in neutral and alkaline solution and this shifted at pH3–3.5 to 305nm (emission 400nm) in acid solution. The absorption spectra of pyridinoline were unchanged at the higher concentrations.

Pyridinoline glycosylation

Freeze-dried tissue samples were hydrolysed in 2M-NaOH (25mg of tissue/ml) in an autoclave at 115°C [70kPa (10lbf/in^2) above atmospheric pressure] for either 6h or 22h. The samples were adjusted to pH4 by the addition of acetic acid and were chromatographed on a column (4cm × 85cm) of Sephadex G-10 with 0.1M-acetic acid as eluent. In some experiments, the hydrolysates were adjusted to pH8 with HCl and were submitted to gel filtration with 0.1M-NH_4HCO_3 as eluent. Fractions containing pyridinoline fluorescence were pooled and evaporated to dryness or freeze-dried.

Chromatography of the fluorescent fraction from a 22h hydrolysate revealed two fluorescent peaks (Fig. 6), one of which was shown to be pyridinoline, and a second, denoted X, that appeared to be less basic. The relative yields of compound X in bovine bone, articular cartilage and Achilles tendon were similar, constituting 15–25% of the total pyridinoline fluoresence. This component reacted with pyridinoline antibody in an enzyme-linked immunoassay (Robins, 1982*b*) performed on samples (10μl) of each fraction of the chromatogram. The pooled fractions containing compound X were desalted by using Bio-Gel P2 as described above. Treatment of

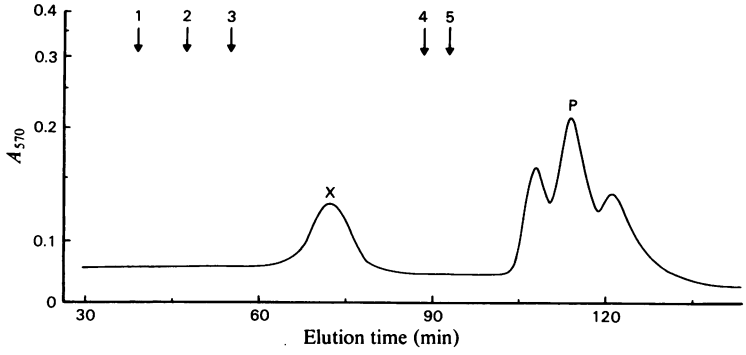

Fig. 6. *Chromatography of pyridinoline derivatives from an alkali hydrolysate*
An alkali hydrolysate of bovine articular cartilage was separated on a Sephadex G-10 column, and the high-M_r fraction was chromatographed with 67mM-sodium citrate buffer, pH4.25, on a 0.9cm × 15cm column of a Locarte analyser. Pyridinoline (P) was eluted as a partially resolved triple peak, and an unknown fluorescent component (X) was eluted at 73min. The arrows indicate the elution positions of reference compounds: 1, glucosylgalactosylhydroxylysine; 2, tyrosine; 3, phenylalanine; 4 and 5, isomers of galactosylhydroxylysine.

compound X with $NaIO_4$ (10mM final concn.) followed by reduction with KBH_4 had no effect on its chromatographic behaviour. On being heated with dilute acid, however, the component was readily converted into pyridinoline; complete conversion was accomplished with 0.1M-HCl at 108°C for 8h, and about 50% conversion was achieved in 1h under these conditions. This sensitivity to acid treatment suggested the avoidance of acid conditions during isolation of the component, but the use of NH_4HCO_3 buffer for the gel-filtration step did not alter the amount of compound X recovered. The compound was, however, also shown to be relatively labile to alkali treatment. Thus hydrolysis of compound X (15.8nmol) in 2M-NaOH at 115°C for 6h resulted in the recovery of only 4.8nmol (30%) of compound X, with much of the remainder (8.7nmol; 55%) present as pyridinoline.

Analysis for hexose in purified compound X was performed essentially as described by Dubois *et al.* (1956) by mixing the sample (50μl) with 6% (w/v) phenol (50μl) and adding conc. H_2SO_4 (300μl). The absorption at 490nm of the cooled solution was measured against a reagent blank. By using purified galactosylhydroxylysine as standard, the measured hexose content of compound X revealed a hexose/pyridinoline molar ratio of 1:0.9, assuming that the ninhydrin colour yield of compound X was identical with that of pyridinoline. After cleavage of the hexose component with 0.1M-HCl as described above, the sample was desalted by passing it successively through columns (0.3cm × 1cm) of Dowex 2 (OH^- form) and Duolite 225 (H^+ form), and then chromatographed on cellulose MN 300 t.l.c. plates with ethyl acetate/acetic acid/water (3:1:3, by vol.; upper phase) as solvent. The hexose derived from compound X co-migrated with a galactose standard on detection with a silver nitrate spray.

On analysis by fast-atom-bombardment mass spectrometry of a solution of compound X in dil. HCl, a peak at *m/z* 558 was the largest mass ion observed in the spectrum, together with a peak at *m/z* 513 corresponding to the loss of a carboxy group.

Discussion

The isolation procedure for pyridinoline developed in the present study differs from that used by Fujimoto *et al.* (1977) in that ion-exchange chromatography was performed on sulphonated polystyrene resins. At low ionic strength and room temperature, pyridinoline is adsorbed strongly on this type of resin and could not, for example, be eluted completely even with 2M-NH_3. Although normal desalting procedures with the use of these resins was therefore impossible, excellent recoveries of pyridinoline were obtained by maintaining column temperatures at 60°C and [Na^+] at 67mM. Similar conditions were used to concentrate the isolated product as a preliminary step to desalting by gel filtration at room temperature.

The structural analysis reported in the present paper confirms the amended structure for pyridinoline proposed by Fujimoto *et al.* (1978). Attempts to obtain mass spectra of pyridinoline as the trifluoroacetyl methyl ester were completely unsuccessful with the use of electron impact or chemical ionization, even though spectrophotometric and other studies indicated that full formation of the derivative had been achieved. Fast atom bombardment is a particularly suitable ionization

technique for this type of compound (Barber *et al.*, 1981), and has recently been used to identify a homologous compound, 3-deoxypyridinoline (Barber *et al.*, 1982). This constitutes a minor component of the pyridinoline fraction isolated by the method of Fujimoto *et al.* (1978), and gave rise to a peak at *m/z* 413 in the mass spectrum. Although a small peak at *m/z* 413 was evident in the present study (Fig. 4), this is unlikely to represent a small amount of the same 3-deoxyderivative in the sample, owing to the method of preparation. The isolation procedure described in the present paper depends on the large differences between pH4 and pH5 in chromatographic behaviour caused by protonation of the ring hydroxy group: clearly, 3-deoxypyridinoline would be basic throughout the pH range 3–6 and would not therefore co-purify in the scheme used. The possibility that an analogue lacking the aliphatic hydroxy group may be present cannot be excluded.

Fluorescence properties

The fluorescence of pyridinoline is linear with concentration up to about 0.1 mM. At higher concentrations, which were encountered during the later stages of purification, the normal fluorescence is fully quenched and entirely different spectra are obtained (Fig. 5). As the absorption spectra remained unaltered, it is probable that this phenomenon is not merely an inner filter effect but is due to the formation of excimers (Stevens & Hutton, 1960), i.e. aggregates of pyridinoline in the electronically excited state. In agreement with this interpretation was the fact that the apparent pK_a of the ring hydroxy group was much lower than that of pyridinoline in dilute solution, a property of the excited state that has been observed for other aromatic alcohols (Whehry & Rogers, 1966). During the initial ion-exchange chromatography step of pyridinoline isolation no characteristic fluorescence could be detected, although the concentration at this stage was much lower than 0.1 mM. It is conceivable that this fluorescence quenching was due to the formation of mixed excimers, possibly with aromatic human-related material that co-chromatographed at that stage.

Glycosylation of pyridinoline

The analysis of compound X from alkali hydrolysates of collagen indicate that it is an *O*-galactosyl derivative of pyridinoline substituted at the side-chain hydroxy group. The expected M_r value of this derivative is 591, whereas the apparent molecular ion peak detected by mass spectrometry was at *m/z* 558: this could be due to dehydration and cyclization of the compound in the spectrometer, but further studies of standard compounds are necessary to clarify this point.

Galactosylpyridinoline was extremely labile to mild acid treatment compared with glycosylated derivatives of bifunctional cross-links (Robins & Bailey, 1974). The isolated compound was also shown to be partially destroyed by further alkali treatment. It is likely, therefore, that hydrolytic degradation of glactosylpyridinoline was the main reason for the low yields of this component that were obtained from tissue hydrolysates. Consequently, the results do not permit any conclusions as to the proportions of glycosylated pyridinoline present in the various tissues analysed. The reason for the alkali-lability may be due to the combined effects of steric factors and the presence of an electron-withdrawing group adjacent to the hexose, which is known to weaken the glycosidic linkage (Overend, 1972). Some evidence for the presence of a disaccharide derivative was obtained, but the yields were insufficient for characterization.

Previous studies have noted the absence of glycosylated derivatives of the tetrafunctional reduction product, histidinohydroxymerodesmosine (Robins, 1982*a*). The factors affecting the stability of pyridinoline glycosides may therefore be relevant to other multifunctional components. Yamauchi *et al.* (1982) were unable to detect any glycosyl derivatives of a trifunctional component, hydroxyaldol-histidine, and attributed this finding to an inhibition by glycosylation of the oxidative reaction necessary to promote formation of the compound. The possible lability of glycosylated hydroxyaldol-histidine to alkaline conditions suggested by the present results could provide an alternative explanation for the results reported by Yamauchi *et al.* (1982).

I am indebted to Dr. Peter Belton, Food Research Institute, Norwich, U.K., for the ^{13}C-n.m.r. spectra and to Dr. Ron Self, Food Research Institute, Norwich, U.K., for analyses by fast-atom-bombardment mass spectrometry.

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