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Chemistry of the collagen cross-links

Origin and partial characterization of a putative mature cross-link of collagen

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The conversion of the reducible divalent cross-links in collagen to non-reducible multivalent cross-links in mature collagen has resulted in the identification of several new amino acids as the putative mature cross-link. None of these compounds has completely satisfied the necessary criteria. We have now isolated an amino acid of high M_r, derived from lysine, that is only present in high-M_r peptides derived from mature collagen. Its increase with age of the tissue correlates with the decrease in the reducible cross-links, and it is present both in mature skin and bone, which are initially cross-linked through the aldimine and oxo-imine divalent cross-link respectively. We propose that this amino acid, as yet incompletely characterized and designated compound M, is a major cross-link of mature collagen.

INTRODUCTION

The inherent strength of collagen, the major structural component of connective tissue, is derived from the extracellular formation of covalent intermolecular cross-links (for reviews see Bailey *et al.*, 1974; Tanzer & Waite, 1982; Eyre *et al.*, 1984). In the early 1970s divalent lysine-derived intermolecular cross-links were demonstrated in type I collagen from various sources. Initial studies established that these cross-links were derived from the interaction of hydroxylysine with either lysine aldehyde to form aldimine bonds (Bailey & Peach, 1968), or with hydroxylysine aldehyde to form oxo-imine bonds (Robins & Bailey, 1973, 1975; Mechanic *et al.*, 1971). Subsequent work showed that these cross-links were also precursors of more complex multivalent intermolecular bonds which formed spontaneously in the tissue during aging (Robins *et al.*, 1973; Robins & Bailey, 1975). It was later proposed that the increased stability of mature collagen was due to the presence of these multivalent cross-links, forming a system of lateral and transverse cross-links in the fibre (for reviews see Light & Bailey, 1979*b*, 1980*a*). Current cross-link research is therefore focused on the nature of these multivalent cross-linking compounds.

Fujimoto and his co-workers isolated a fluorescent hydroxypyridinium derivative from mature collagen which was given the trivial name of pyridinoline (Fujimoto *et al.*, 1977). This amino acid, the product of reaction of a hydroxylysine aldehyde with the oxo-imine cross-link, was proposed as the major stabilizing trivalent moiety of the mature collagen matrix (Fujimoto, 1980; Eyre & Oguchi, 1980). However, the collagen in certain tissues such as skin does not contain the oxo-imine cross-link and cannot therefore form pyridinoline. The maturation route of the aldimine cross-link in these tissues has not yet been elucidated, although it has been suggested (Eyre *et al.*, 1984) that it may be converted to hydroxyaldolhistidine, previously identified by Housley *et al.* (1975).

Adopting a different approach, we have isolated an oligomeric peptide complex from CNBr digests of mature collagen. This cross-linked complex was named poly-α1CB6 because the major peptide component was shown to be the large α1CB6 peptide attached to the small α1CB5 (Light & Bailey, 1979*a*, 1980*b*). Poly-CB6 did not contain pyridinoline when extracted from skin, tendon or bone, the latter two tissues containing significant amounts of pyridinoline in a total acid hydrolysate. The role of pyridinoline as the major stabilizing cross-link in mature collagen must therefore be questioned.

In the present paper we report the identification of an unusual amino acid derived from acid hydrolysates of poly-α1CB6. This compound has a higher M_r than normal amino acids, is derived from lysine as shown by incorporation experiments *in vivo* and is only present in high-M_r cross-linked peptides derived from mature collagen. We propose that this amino acid moiety is a true mature cross-link of collagen.

MATERIALS AND METHODS

Materials

[^{3}H]Lysine (40 Ci/mmol), [^{3}H]valine and [^{14}C]leucine were obtained from Amersham International. All other reagents were analytical or h.p.l.c. grade. Sephadex G-10 was from Pharmacia. Zeolit 225 and Fractogel TSK-HW40(s) were from BDH. Zorbax ODS (5 μm) and Zorbax-NH_2 (5 μm) were from Du Pont. Techsil C_{18} (5 μm) was obtained from HPLC Technology, Macclesfield, Cheshire, U.K. All salts, including Fluram, were AnalaR grade from BDH. All organic solvents and heptafluorobutyric acid were h.p.l.c. grade from Rathburn Chemicals, Walkerburn, Peebleshire, Scotland, U.K. Mature bovine tendons were obtained at slaughter and were either treated immediately or stored at -20 °C until use.

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Preparation of hydrolysates

Samples of peptides, whole collagen molecules and tissues were hydrolysed in 6 M-HCl at 110 °C under air for 36 h. Hydrolysates were rotary evaporated, redissolved in water, filtered, re-evaporated and then stored dry at −20 °C. For amino acid cross-link analysis, a preliminary step of gel filtration was introduced to remove low-M_r amino acids. The hydrolysates were dissolved in water, neutralized with NH_4OH and chromatographed on Sephadex G-10 columns (95 cm × 1.6 cm or 140 cm × 5 cm) in 0.05 M-acetic acid. [^{3}H]Lysine was added to each sample as an internal M_r marker and, after locating the lysine peak by scintillation counting, all material eluting before the marker was pooled, rotary evaporated and resuspended in a known volume of 1 mM-HCl. Samples were analysed on either a Jeol 6AH or an LKB 4400 amino acid analyser, with a sodium citrate buffer system.

SDS/polyacrylamide-gel electrophoresis

For analysis of CNBr and thermolysin fragments the method of Laemmli (1970) was used as described by Light (1982). Analysis of whole collagen α-chains used the method of Sykes & Bailey (1971). All gels were stained with Coomassie Brilliant Blue and destained before photography and densitometry.

Preparation of the putative cross-link compound from poly-α1CB6

Poly-α1CB6 was prepared from mature bovine tendons by CNBr digestion, gel filtration and ion-exchange chromatography as previously described (Light & Bailey, 1980*c*) and then hydrolysed as previously described. The hydrolysate was subjected to gel filtration on Sephadex G-10 to remove low-M_r amino acids as before. The pre-lysine material was taken up in 0.1 M-pyridine/formate buffer, pH 2.9, and subjected to ion-exchange chromatography in pyridine/formate buffers as previously described (Light & Bailey, 1982). The material eluting between marker peaks of [^{3}H]valine and [^{14}C]leucine was pooled, dried under vacuum and redissolved in 0.2 M-sodium formate, pH 2.6. This material was then chromatographed on a column (23 cm) of the Jeol 6AH amino acid analyser modified for preparative work and equilibrated in the same buffer at 55 °C. The column was eluted with 0.2 M-sodium formate buffers: pH 2.6 (40 min), pH 3.0 (40 min) and pH 4.0 (40 min).

The major amine peak, denoted compound 'M' (for mature cross-link) eluting at 100 min between the markers valine and leucine was pooled and rotary evaporated to dryness at 50 °C.

The dry residue was taken up in 0.05 M-acetic acid and desalted on a Sephadex G-10 column (25 cm × 2.5 cm) in the same solvent. The sample was dried, redissolved in 0.5 ml water and subjected to reverse-phase h.p.l.c. on a column (25 cm × 0.46 cm) of Zorbax ODS (5 μm) at room temperature and at 1 ml/min. The column was eluted with water and compound M was not retained. The unbound material was dried, taken up in 0.5 ml of 70% (v/v) methanol, loaded on a column (25 cm × 0.46 cm) of Zorbax-NH_2 at 35 °C and eluted with acetonitrile/water (77:23, v/v) at 2 ml/min. The material was finally chromatographed on Bio-Gel P-2 (BioRad) in 0.05 M-acetic acid.

Large-scale preparation of the compound M

To obtain sufficient compound M for chemical analysis, 200 g batches of aged bovine Achilles tendons were cleaned and hydrolysed as before. The hydrolysate was filtered, rotary evaporated to dryness six times and re-dissolved in a sufficient volume of 0.2 M-NaOH (approx. 1 litre) to give a final pH of 2.3. This was then applied to an ion-exchange column consisting of Duolite 225 SRC 16 resin (5 cm × 25 cm) at 55 °C. The column was eluted with 4 litres of 0.2 M-sodium formate buffer, pH 3.0, followed by 2 litres of formate buffer, pH 4.0, and the effluent was monitored at 257 nm.

The fractions corresponding to the pH 4.0 ion front were pooled and rotary evaporated to 50 ml. The sample was applied to a Sephadex G-10 gel filtration column as before. The pre-[^{3}H]lysine fractions were pooled, rotary evaporated to dryness and applied in three batches to a 45 cm ion-exchange column on the modified Jeol JLC 6AH amino acid analyser. The column was eluted sequentially with pH 3.0 and pH 4.0 sodium formate buffers (0.2 M). The presence of amines in each fraction was determined by measurement of fluorescence after reaction with Fluram (Weigele *et al.*, 1972). The peak eluting at the pH 4.0 ion-front was collected and freeze-dried.

Further purification of the relevant peak was achieved by size-exclusion chromatography on a column (95 cm × 1.5 cm) of Fractogel 40(s) eluted with 0.05 M-acetic acid at a flow rate of 0.4 ml/min. The major amine peak was identified by assaying aliquots of collected fractions with Fluram. The pooled fractions were freeze-dried, redissolved in 1% heptafluorobutyric acid and subjected to ion-pair h.p.l.c. on a Techsil C_{18} column (25 cm × 0.46 cm) eluted with a gradient of 5–15% (v/v) methanol in 0.01 M-heptafluorobutyric acid. The major amine peak, eluting as a doublet at 10% methanol, was identified by Fluram assay as shown in Fig. 4. The peaks were collected, freeze-dried and stored at −20 °C until required.

Incorporation of [^{3}H]lysine into rabbit collagen

A 3 month New Zealand White rabbit was given five consecutive daily subcutaneous injections of 4 mCi of [^{3}H]lysine in phosphate-buffered saline (0.15 M-NaCl containing 0.02 M-sodium phosphate buffer, pH 7.4), and 14 days after the first injection it was killed by exsanguination. The tendons, long bones and skin were collected immediately and were processed as quickly as possible. Where necessary, samples of tissue were stored at −20 °C before use.

The skin, freed from hair, was finely minced and extracted with phosphate-buffered saline. A portion of the skin was set aside for further investigation, and the remainder was extracted overnight with 1% (w/v) SDS containing 50 mM-Tris/HCl buffer, pH 7.4. The skin was then exhaustively washed in water prior to acid hydrolysis.

Bone was decalcified by washing in 0.75 M-EDTA, pH 8.5, with five changes of wash solution. After washing the bone chips in water they were blot-dried and hydrolysed.

Compound M was prepared from each tissue (5–10 g) by the methods described above and was assayed both by standard amino acid analysis on the Jeol 6AH as well as by h.p.l.c. on the Zorbax-NH_2 column.

Acid-soluble collagen was extracted from the remaining untreated rabbit skin with 0.5 M-acetic acid for 16 h at 4 °C, and SDS-soluble collagen was extracted from the residue with the SDS buffer described above for 24 h at 20 °C. Collagen was precipitated from the acid extract by the addition of 10% (w/w) NaCl. The SDS extract was dialysed against water and was freeze-dried before hydrolysis.

CNBr peptides of collagen from the SDS-washed insoluble skin residue were also prepared by the method previously described (Light & Bailey, 1980*b*,*c*) and were separated on a basis of M_r by gel filtration on an agarose A1.5m column (95 cm × 5 cm) in 1 M-$CaCl_2$/50 mM-Tris/HCl, pH 7.4. Peptide pools were dialysed against water and freeze dried.

Assay of compound M in tissue hydrolysates

Purity and concentration. The purity and approximate concentration of compound M in hydrolysates which had been prepared as described was assayed using either the Jeol 6AH or LKB 4400 amino acid analyser. The Jeol assay was carried out on a 25 cm column heated to 60 °C at a flow rate of 1.1 ml/min, using 0.2 M-sodium citrate buffers at pH 3.1 for 90 min and at pH 4.0 for 40 min. Using this system the compound eluted as a symetrical peak at 111 min. Assayed on the LKB analyser using a standard elution system, compound M eluted as a sharp peak just prior to the elution position of isoleucine.

Thin layer chromatography. Standard amino acids and compound M preparations, both crude and highly purified, were separated by t.l.c. on Polygram Cel 300 cellulose plates in propan-2-ol/butan-2-one/1 M-HCl (12:3:5, by vol.). Plates were developed for 3.5 h at room temperature, at which time the solvent front had migrated between 10 and 14 cm. The mobilities of compound M and standards, stained by spraying with 1% (w/v) ninhydrin in acetone and incubating at 80 °C for 30 min, were measured and calculated as R_F values relative to the mobility of alanine.

High voltage paper electrophoresis. This was carried out on 40 cm × 18 cm strips of 3MM Whatman chromatography paper in a Camag water-cooled apparatus. Runs were carried out at pH 1.9 in 1.8% (v/v) formic acid for 1 h at 2.5 kV and 10 mA or at pH 6.5 in pyridine/acetic acid/water (25:1:225, by vol.) for 1 h at 2 kV and 30 mA. Ninhydrin-positive material was located by spraying the paper, dried after electrophoresis at 40 °C, with 1% (w/v) ninhydrin in acetone and heating to 80 °C for 30 min.

Estimation of M_r. An approximate M_r was determined against known standards by size-exclusion chromatography on a Fractogel 40 s column using elution conditions as described above.

Mass spectrometry. Samples were analysed by fast atom bombardment mass spectrometry on a M59/50 Kratos instrument. A solution of unmodified compound M in 0.05 M-acetic acid was applied directly to a probe coated in thioglycerol. The sample was analysed using both positive and negative ion mode.

Age-related changes in reducible cross-links and compound M

Aging *in vitro*. Portions of the minced [^{3}H]lysine-labelled rabbit skin were also aged *in vitro* by incubation at 37 °C in well-oxygenated phosphate-buffered saline (containing 0.02 M-NaN_3 as a bacteriostat) for 7, 14, 21 and 35 days. At the end of each incubation period the sample was divided into two equal portions (11 g wet wt. each) and one was reduced with KBH_4 at room temperature for 1 h at a 100-fold molar excess of the reagent over substrate. Both portions were then digested with CNBr as described and the soluble fraction from each was retained, dried and hydrolysed for analysis.

Aging *in vivo*. Sheep tendons from animals of various ages (newborn, and 8, 16, 20, 24 and 52 weeks) were analysed for the changes both in the reducible cross-links and in compound M.

RESULTS

Preparation of compound M

The procedures used to identify and purify the cross-link are summarized in Fig. 1. Compound M was initially isolated from poly-α1CB6 by gel filtration, ion-exchange chromatography, reverse-phase and normal-phase h.p.l.c. Gel filtration of the hydolysates on Sephadex G-10 in 0.05 M-acetic acid showed the compound M distributed as a broad peak (as assayed on the Jeol amino acid analyser), eluting just before the [^{3}H]lysine marker peak. The relevant tubes were pooled and subjected to a bulk ion-exchange chromatography step in volatile pyridine buffers. The relevant fractions, identified by [^{3}H]valine and [^{14}C]leucine markers, were pooled, rotary evaporated and re-chromatographed in sodium formate buffers on a modified 23 cm Jeol ion-exchange column at 55 °C. Compound M eluted as a relatively sharp symmetrical peak late in the run and was pooled and reduced to dryness by rotary evaporation.

After desalting on a short Sephadex G-10 column the sample was eluted from a Zobax C18 column which absorbed out standard amino acid contaminants and non-amine compounds derived from the hydrolysates and the ion-exchange columns. The effluent from the reverse-phase h.p.l.c. column was dried and taken up in 70% (v/v) methanol prior to chromatography in 77% (v/v) acetonitrile on a Zorbax-NH_2 column at 35 °C. Compound M eluted as a single peak at 72 min. Considerable overlap of the major peaks was seen if the column was overloaded and so, in some cases, repetitive runs were required.

The Zorbax-NH_2 column was found to leach off 2-propylamine during use (Smolenski *et al.*, 1983), so the final compound M product was subjected to gel filtration on Bio-Gel P-2 in 0.05 M-acetic acid to remove low-M_r contaminants.

The same component was isolated and purified from mature bovine tendon hydrolysates by the modified procedure using ion-exchange chromatography on a large Duolite column, followed by preparative chromatography on the Jeol amino acid analyser. Compound M eluted as a sharp peak from the Jeol analyser 30 min after the start of the pH 4 buffer (i.e. 120 min).

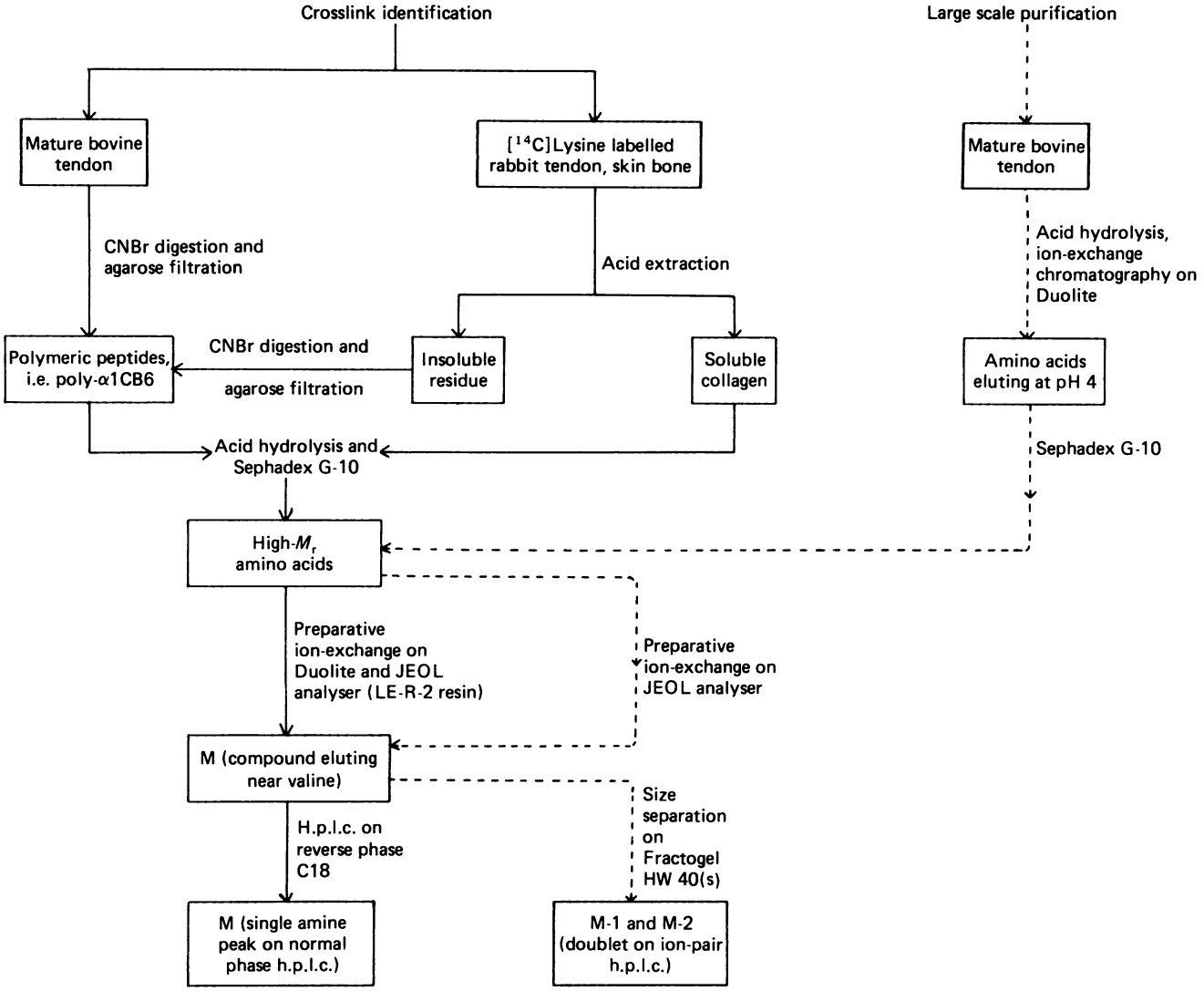

Fig. 1. Flow diagram of preparative procedures

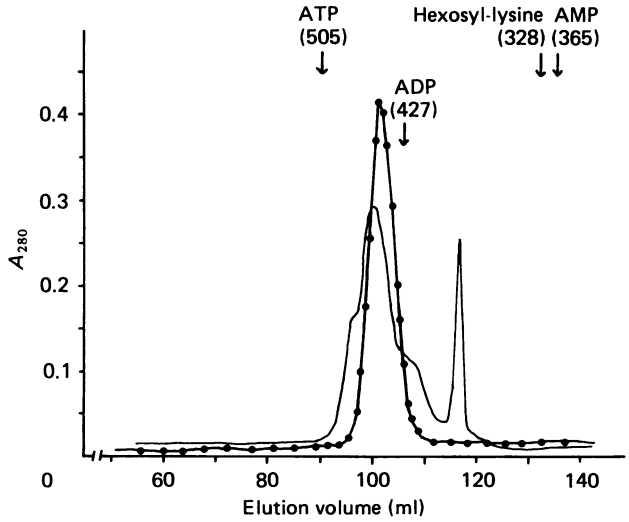

Fig. 2. Purification and M_r determination of compound M on Fractogel HW 40(s) size-exclusion column

The solid line indicates A_{280}. Compound M was assayed by reactivity with Fluram (●). Elution volumes of M_r standards are indicated by arrows.

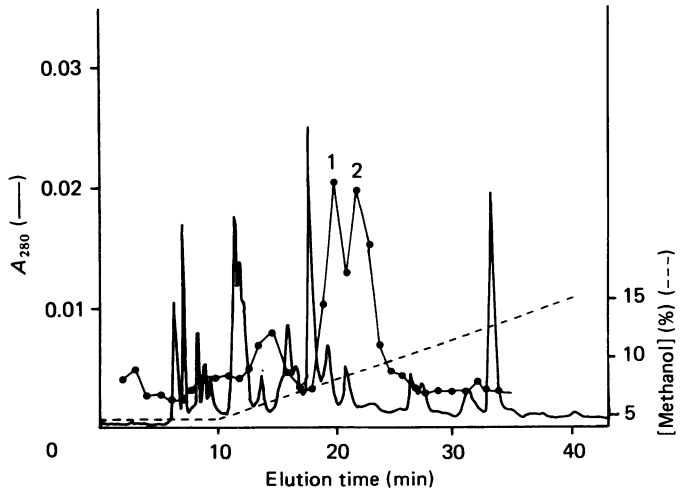

Fig. 3. Purification of compound M by ion-pair reverse-phase h.p.l.c.

——. A_{280}; – – –, solvent gradient; ●, cross-link assayed by reactivity with Fluram. Peaks 1 and 2 were collected for analysis.

Subsequent size-exclusion chromatography on Fractogel 40(s) gave a major amine peak eluting with an approximate M_r of 460, as seen in Fig. 2. This sample was subjected to ion-pair h.p.l.c. as shown in Fig. 3. The component M was resolved into two closely eluting peaks, M-1 and M-2, which were collected separately and analysed by mass spectrometry and t.l.c.

Thin layer chromatography

T.l.c. of the compound M showed it to be a unique amine which migrated on cellulose plates with an R_F of 0.1 relative to alanine. The compound migrated very slowly as a single spot barely leaving the origin and considerably slower than neutral, hydrophobic and acidic amino acid standards. Both M-1 and M-2 migrated with the same R_F value, suggesting that the compound had been resolved into isomeric forms on the ion-pair h.p.l.c. system.

Mass spectrometry

On analysis by fast atom bombardment mass spectrometry, the positive ion spectrum of both M-1 and M-2 showed a major ion at m/z 447. There appeared to be few fragmentation products, but this is not unusual with this form of mass spectrometry. In negative mode the ion spectrum showed a major ion at m/z 445. The two spectra indicate isomers of a molecule of mass 446 migrating as $M+1$ in the positive mode and $M-1$ in the negative mode. No lysine-derived cross-linking amino acid has been reported with a mass of 446.

The sample gave no spectrum on either desorption chemical ionization or electron impact ionization mass spectrometry.

High voltage paper electrophoresis

High voltage paper electrophoresis of the compound at pH 1.9 and pH 6.5 showed it to migrate as a single component. At pH 1.9 compound M migrated with a mobility of 0.88 relative to alanine, a value similar to that of a desmosine standard. From the work of Bailey & Ramshaw (1973) it was possible to calculate an approximate value for the charge carried by compound M at this pH, and a value of +2.6 was obtained. This implies that compound M is a complex amino acid.

Incorporation of [^{3}H]lysine into compound M from rabbit collagen

The highest incorporation of [^{3}H]lysine per gram of dry tissue was seen in the rabbit skin. Bone also showed a high incorporation but tendon, which has a long turnover time, was low (5-fold lower than skin) and did not contain sufficient activity for further analysis.

The skin and bone hydrolysates were subjected to gel filtration and ion-exchange chromatography as described in the Materials and methods section. The resulting chromatograms are shown in Fig. 4. There was a strong radioactive peak separated by ion-exchange chromatography from the bone hydrolysate which was also highly fluorescent and was identified as pyridinoline (Fig. 4*b*). However, in both skin and bone there appeared only two other peaks of radioactivity (both approximately the same size as pyridinoline, indicating that these compounds are present in the same proportionate degree), peak 1 eluting with isoleucine and peak 2 just before hydroxylysine. No other major peaks of radioactivity

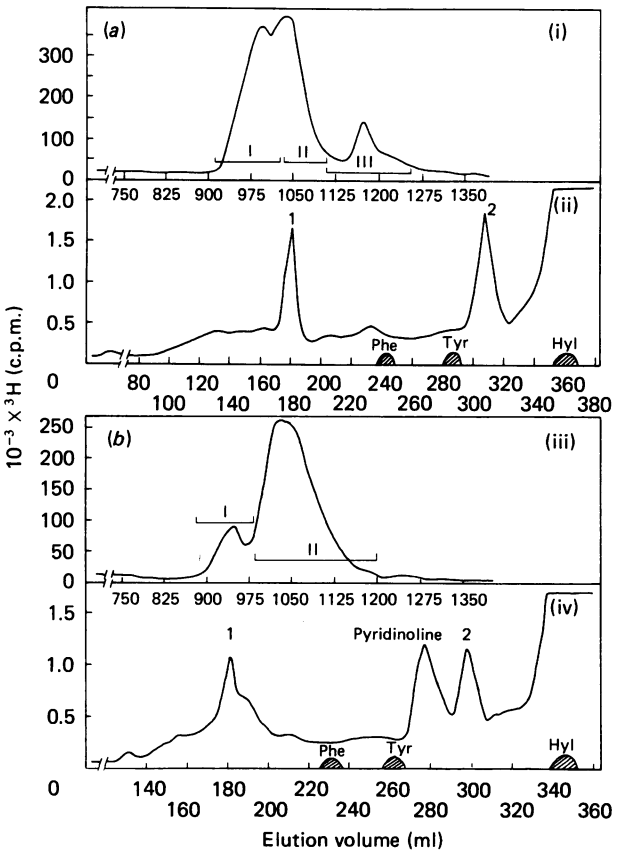

Fig. 4. Preparation of compound M from rabbit tissues labelled with [^{3}H]lysine *in vivo*

(*a*) Skin, (*b*) bone. Acid hydrolysates of rabbit tissue labelled *in vivo* with [^{3}H]lysine were obtained as described in the Materials and methods section. (i) and (iii), gel filtration of the hydrolysate on Sephadex G-10. The fractions denoted by bars were pooled and fraction I was taken on to ion-exchange chromatography on Duolite 225 in pyridine/formate buffers. (ii) and (iv), ion-exchange preparative chromatography of fraction I from above. Fractions 1 and 2 were stored for further analysis.

were seen. Peak 1 from the skin hydrolysate chromatographed in the same region as pure compound M on the preparative pyridine/formate columns. When this peak was re-chromatographed on the Jeol 6AH amino acid analyser a major peak of radioactivity co-migrated with pure compound M. When peak 1 from the bone hydrolysate was similarly run on the Jeol 6AH an identical result was obtained.

The identity of peak 1 as compound M was confirmed by chromatographing it on the h.p.l.c. Zorbax-NH_2 column. In this case the major peak of radioactivity again co-migrated with genuine compound M. These two pieces of evidence show that the main labelled compound in peak 1 from rabbit bone and skin was compound M and that it was derived from lysine.

The second compound, peak 2, eluting after phenylalanine on the pyridine/formate system, was analysed on the long basic column of the Jeol analyser and was shown to migrate with lysine. It had an M_r by gel filtration of approx. 500 but was not fluorescent. Although this material may be suspected as a cross-link of collagen from its high M_r, it was not present in a CNBr mixture

obtained from the rabbit skin that contained only pure collagen peptides (results not shown).

When CNBr peptides of the rabbit skin collagen were separated by gel filtration and analysed after acid hydrolysis for compound M content it was found that the greater proportion of the compound was associated with peptides in the M_r range 15000–50000 (i.e. cross-linked polymers). The M_r is lower than for bovine skin collagen since α1CB6 in rabbit skin is smaller. In addition, acid-extracted rabbit skin collagen, which was shown by SDS/polyacrylamide-gel analysis to contain no cross-linked oligomeric complexes at the top of the gel, contained no detectable compound M. In contrast, SDS-extracted collagen, which showed the presence of some high-M_r oligomers on SDS gels, did contain a low level of the compound, 0.21 nmol/mg of collagen. Finally, the highest level of compound M content per mg of collagen was found in the acid- and SDS-insoluble collagen of the skin residue, 1.57 nmol/mg of collagen, equivalent to 0.47 mol of cross-link/mol of collagen.

Aging of rabbit skin *in vitro*

Rabbit skin was aged *in vitro* in phosphate-buffered saline at 37 °C for up to 35 days to assess the formation of compound M. By reducing half of each sample and analysing it separately we were able to assess the efficacy of the method by following the disappearance of reduced aldimine, the bivalent precursor cross-link (Fig. 5). As can be seen, an approximately exponential decrease in the amount of this compound was observed as previously reported for aging both *in vivo* and *in vitro* (Robins *et al.*, 1973; Robins & Bailey, 1977). In parallel with this decrease in aldimine we saw an increase in compound M which closely resembled the theoretical mirror image of the curve for aldimine. This evidence suggests that compound M is formed from aldimine precursors in skin.

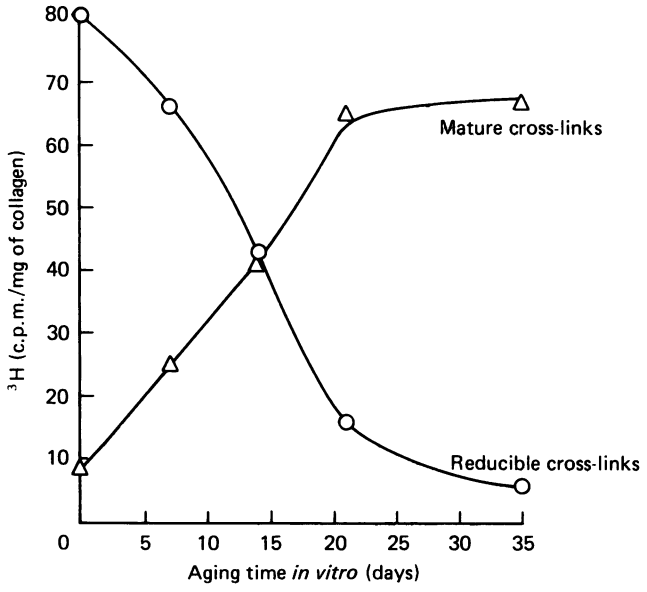

Fig. 5. Aging of rabbit skin *in vitro*

Rabbit skin labelled *in vivo* with [^{3}H]lysine was incubated at 37 °C for up to 35 days *in vitro*. Samples were taken at 7, 14, 21, 28 and 35 days and were assayed before and after reduction with KBH_4 for compound M (△) and for the aldimine cross-link hydroxylysinonorleucine (○) respectively.

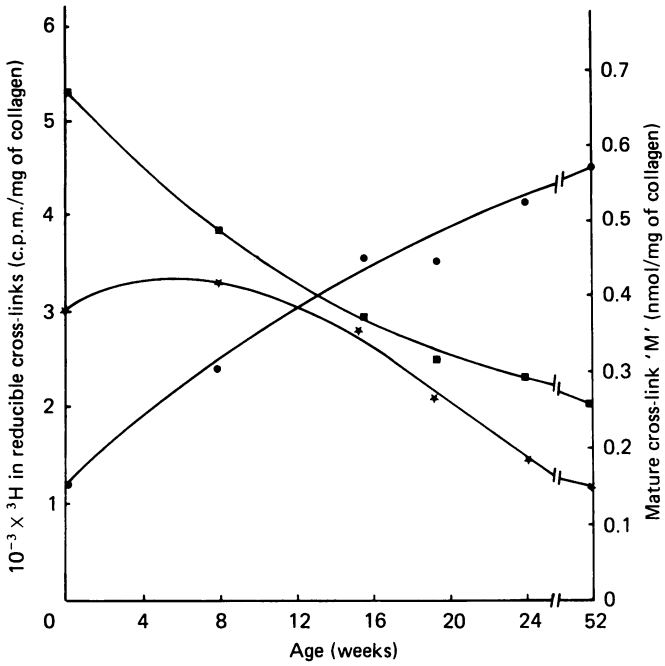

Fig. 6. Aging of sheep tendon *in vivo*

Tendons from sheep of various ages (newborn, and 8, 16, 20, 24 and 52 weeks) were acid-hydrolysed and assayed for the content of reducible cross-links and compound M. ★, aldimine; ■, oxo-imine; ●, compound M.

Aging *in vivo*

The decrease of the two reducible divalent cross-links was followed over 52 weeks in tendon from sheep of various ages. It is clear from Fig. 6 that the decrease in the aldimine and oxo-imine cross-links paralleled the increase in the concentration of compound M.

DISCUSSION

The identification of a new amino acid of high M_r derived from lysine and only present in mature collagenous tissues leads us to propose that it is the elusive 'mature' cross-link of collagen.

This compound, termed M, has now been partially characterized. It is associated only with high-M_r cross-linked peptides derived from insoluble mature collagen. This evidence alone strongly suggests that the component is the covalent cross-link in these peptides. However, further proof was needed to confirm its role as a cross-link.

Chromatographic analysis of rabbit collagen after incorporation of [^{3}H]lysine *in vivo* revealed that compound M was a post-biosynthetic derivative of polypeptide lysine in collagen, and therefore probably based on the precursor lysine-aldehyde. These studies also demonstrated the specific location of the compound in insoluble collagen and in complex, cross-linked CNBr peptides. No other radiolabelled lysine derivatives (except pyridinoline in bone) were observed in purified insoluble collagen from the rabbit skin and bone.

When rabbit skin labelled with [^{3}H]lysine *in vivo* was allowed to age *in vitro*, a direct correlation was found

between the decrease in the quantity of bifunctional aldimine cross-links (long suggested to be the precursors of the more complex mature cross-links; see Robins & Bailey, 1973; Light & Bailey, 1980*a*) and the increasing content of compound M in the skin collagen. This final evidence for the role of compound M as a mature cross-link in collagen is extremely important. Not only does the compound appear only in cross-linked material, showing that it cannot be merely an acid-produced lysine artefact of hydrolysis, but also its increasing concentration in aging tissue can be seen to be directly linked to the disappearance of the supposed precursor of the mature cross-link.

Another important consideration is that compound M was also found, in quantity, in bone, a tissue which has recently received considerable attention and in which pyridinoline was proposed as the major mature cross-link. Recent work by Robins & Duncan (1983) on cartilage type II collagen and from our own laboratory (Light & Bailey, 1985) on tendon type I collagen has shown that pyridinoline is not involved in the stabilization of the polymeric complexes which must be largely responsible for the high rigidity, insolubility and strength of mature collagen fibres. Instead, it appears that this cross-link forms as a bridge probably only between two molecules, thus adding no more intrinsic strength than the initial bifunctional oxo-imine bonds. This suggests that pyridinoline does not have an important role in stabilizing mature collagen in the way in which we expect, that is through the lateral cross-linking of 'microfibrils' in register (Bailey *et al.*, 1980).

The presence of compound M in bone indicates that the cross-link is the product of a similar mechanism of maturation occurring in bone as well as in skin. Calculation of the approximate relative quantities of the compound in both skin and bone revealed that these were almost equal, showing that the mature cross-link is important as a stabilizing cross-link of collagen in both tissues. Obviously, as the predominant precursor of the mature cross-link in skin is the aldimine as opposed to the oxo-imine in the bone, compound M must be formed from both precursors by a similar mechanism to form closely related compounds.

The absence of pyridinoline from skin collagen has prompted Eyre *et al.* (1984) to propose that there are two pathways in the maturation of collagen, one for tissues possessing the precursor lysine aldehyde, e.g. skin, rat-tail tendon, cornea and sclera, and a different pathway leading to pyridinoline for those possessing hydroxylysine-aldehyde. Eyre has further suggested that the former pathway evolved in the case of tissues exposed to u.v. light, that is skin and cornea, owing to the instability of pyridinoline in u.v. light. However, many tissues, e.g. rat-tail tendon and fish skin, do not contain pyridinoline and are not exposed to u.v. light. The nature of the mature cross-link in these tissues has not been elucidated, but Eyre has suggested evidence for the formation of hydroxyaldol histidine previously isolated from mature skin by Housley *et al.* (1975). An additional problem is that a number of tissues are cross-linked by the oxo-imine via the hydroxylysine aldehyde but do not possess pyridinoline, for example basement membrane collagen. The cross-linking of these tissues would require a third mechanism to provide a mature cross-link.

In contrast we have shown that the mature cross-link compound M is present to a significant extent in skin which is initially stabilized by the aldimine, in bone which is stabilized by the oxo-imine and pyridinoline, and in basement membrane which is initially stabilized by the oxo-imine but does not possess pyridinoline. The presence of this as yet incompletely characterized cross-link in these mature tissues suggests a common mechanism for the maturation of all collagens.

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