

Chemistry of the Collagen Cross-Links

ISOLATION AND CHARACTERIZATION OF TWO INTERMEDIATE INTERMOLECULAR CROSS-LINKS IN COLLAGEN

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This paper describes the isolation from reduced collagen of two new amino acids believed to be involved, in their non-reduced form, as intermolecular cross-links stabilizing the collagen fibre. The reduction of intact collagen fibrils with tritiated sodium borohydride was found to stabilize the aldehyde-mediated cross-links to acid hydrolysis and thus allowed their location and isolation from acid hydrolysates on an automatic amino acid analyser. Comparison of the radioactive elution patterns from the autoanalyser of collagen treated in various ways before reduction permitted a preliminary classification of the peaks into cross-link precursors, intramolecular and intermolecular cross-links. The techniques employed to isolate the purified components on a large scale and to identify them structurally are described in detail. Two labile intermolecular cross-links were isolated in their reduced forms, one of which was identified by high-resolution mass spectrometry as *N*^ε-(5-amino-5-carboxypentyl)hydroxylysine. The structure of this compound was confirmed by chemical synthesis. The cross-link precursor α -amino adipic δ -semialdehyde was isolated in its reduced form, ϵ -hydroxynorleucine, together with its acid degradation product ϵ -chloronorleucine. A relatively stable intermolecular cross-link was isolated and partially characterized by mass spectrometry as an aldol resulting from the reaction of the δ -semialdehyde derived from lysine and hydroxylysine.

The function of collagen is mainly mechanical and it is generally accepted that the collagen fibres are stabilized by a system of covalent interchain cross-links (for reviews see Bailey, 1968*b*; Veis, 1967). From a functional point of view it is unlikely that the cross-links within the tropocollagen molecule (Bornstein & Piez, 1966) contribute to the stability of the intact fibre, but rather the intermolecular bond, since it must be this bond that prevents slippage of adjacent molecules. The ability of lathyrogens to render collagenous tissue extremely fragile by inhibiting the biosynthesis of all the cross-links strongly suggested a common biosynthetic route, involving the lysine-derived aldehydes, for both the intramolecular cross-link of the collagen molecule (Piez, 1968) and the intermolecular cross-links stabilizing the collagen fibre.

Recent studies have demonstrated that in addition to the intermolecular cross-links stable to weak acids and thermal denaturation (Veis & Anesey, 1965) young native collagen also contains a proportion of labile intermolecular cross-links, and it was proposed that they involve an aldimine bond (Bailey, 1968*a*). A preliminary account of the

identification of one of these aldimine bonds in rat tail tendon has been reported (Bailey & Peach, 1968). In young rat tail tendon the reducible intermolecular cross-links were predominantly labile, thus accounting for its ready solubility. The achilles tendons from both rats and bovines are much less soluble and must therefore contain additional intermolecular cross-links relatively more stable to acid extraction procedures. The present paper describes the isolation and identification of two intermolecular cross-links in mammalian achilles-tendon collagen, one being relatively stable whereas the other is a labile aldimine bond.

MATERIALS AND METHODS

Materials

Zeo-Karb 225 as obtained from the manufacturers (The Permutit Co. Ltd., London W.4, U.K.) was washed with 2*M*-NaOH and 2*M*-HCl before use. DL-5-Hydroxylysine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Chemicals for Bray's (1960) solution were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. Pyridine (AnalaR) was obtained from

BDH Chemicals Ltd., Poole, Dorset, U.K., and before use refluxed with ninhydrin (2g/l) for 30 min and then collected by distillation. Universal Buffer Mixture was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. β -Aminopropionitrile was supplied by Ralph Emmanuel Ltd., London S.E.1, U.K.

L-[4,5- ^3H]Lysine monohydrochloride (250 mCi/mmol), L-[U- ^{14}C]lysine monohydrochloride (300 mCi/mmol) and sodium borohydride (400 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Methods

Preparation of intact collagen fibres. Bovine achilles tendons were obtained from 1–4-week old calves, dissected free of adhering fat and muscular tissue, shredded in an MSE Ato-Mix homogenizer and washed with copious amounts of 0.9% NaCl, pH 7.4. Leg tendons from 9-month-old Sprague-Dawley [Carworth (Europe), Huntingdon, Hunts., U.K.] rats and from 1-day-old chicks (White Leghorn stock) were treated in a similar manner.

Incorporation of labelled lysine. Fertilized eggs (White Leghorn stock supplied by F. G. Sykes Ltd., Bridgwater, Somerset, U.K.) were incubated for 15 days at 39°C. They were then injected onto the chorioallantoic membrane with L-[U- ^{14}C]lysine (0.05 mCi) or L-[4,5- ^3H]lysine (0.2 mCi). Incubation was continued for a further 5 days and the embryos were then killed and the leg tendons removed.

Preparation of lathyrin collagen. The 15-day-old incubated fertilized eggs obtained as above were made lathyrin by chorioallantoic injection of various doses (0.1–0.4 ml) of a sterilized solution of β -aminopropionitrile (100 mg/ml) in buffered saline (0.9% NaCl, pH 7.4). After 48 h further incubation at 39°C the embryos were killed and the leg tendons removed.

Isolation of N-terminal telopeptide. Purified tropocollagen solutions in 0.05% acetic acid were treated with pepsin to make an enzyme/substrate weight ratio of 1:100 (Drake, Davison, Bump & Schmitt, 1966). The mixture was gently agitated for 12 h at 18°C and then dialysed against four changes of 0.05% acetic acid for 24 h. The diffusate was concentrated on a rotatory evaporator *in vacuo*. The pepsin in the dialysis residue was inactivated by raising the pH to 8.0. The precipitate was redissolved and precipitated by addition of KCl to a final concentration of 15% (w/v), and again dissolved in 0.05% acetic acid. A portion of this solution was then checked for complete removal of the β components by electrophoresis on polyacrylamide gels by using a modification (Fessler & Bailey, 1966) of the method of Nagai, Gross & Piez (1964).

Soluble collagen. Tropocollagen solutions were prepared from rat tail tendon and bovine achilles tendon by extraction with 0.05% acetic acid and purified by repeated salt [5% (w/v) NaCl] precipitation and centrifugation, and a final ethanol (30%, v/v) precipitation.

Reduction of collagen fibres. The fibres were suspended in 0.9% NaCl buffered to pH 7.4 with carbonate or Universal buffer, and solid NaB^3H_4 , diluted with non-radioactive borohydride to approx. 10 mCi/mmol, was added directly to the stirred suspension to give a collagen/borohydride ratio of 30:1 (w/w) based on the dry weight of

collagen (approx. 200-fold molar excess of NaB^3H_4). When comparisons were made between the reduction of different tissues, the NaB^3H_4 was first dissolved (50 mg/ml) in saline-carbonate buffer or Universal buffer, pH 7.4, at 5°C and then equal volumes of the solution were added to the various suspensions of fibres to give a final ratio of 30:1. The reduction was allowed to proceed at room temperature for 1 h, stopped by addition of acetic acid to lower the pH to 4.0, and the suspension dialysed for 24 h against several changes of water. The fibres were then freeze-dried and hydrolysed.

Reduction of soluble collagen and isolated α -chains. Reduction of tropocollagen solutions at pH 7.4 was carried out in the presence of 0.1 M-calcium acetate to prevent precipitation as fibrils. Gelatin solutions were reduced in Universal buffer, pH 7.4, by addition of a solution of NaB^3H_4 (200-fold molar excess) for 1 h at room temperature; the solution was acidified to pH 4.0 and then extensively dialysed as above. The component α -chains were isolated on CM-cellulose (Whatman CM-52) by using the procedure of Piez, Eigner & Lewis (1963) and reduced as described above.

Hydrolysis procedure. The reduced collagen samples were hydrolysed by refluxing with freshly redistilled constant-boiling HCl for 24 h. The HCl was removed from the hydrolysate by evaporation *in vacuo*.

Isolation of radioactive components from the hydrolysate of reduced collagen

Displacement chromatography of acid hydrolysate. Stage 1: cation exchange. The technique was based on the system designed by Partridge & Brimley (1952) for the separation of the standard amino acids on a large scale. Three columns, 7.6 cm \times 56 cm, 5.1 cm \times 50 cm and 3.8 cm \times 47 cm, were filled with Zeo-Karb 225 (8% divinylbenzene; H^+ form; 100–200 mesh) and connected in series. Coupled columns of diminishing diameter were employed to improve the sharpness of the final boundaries. The concentrated hydrolysate from 250 g of reduced collagen was diluted and when necessary adjusted to pH 1.0 with HCl, and 25 g of purified pyridine was added to increase the separation between the neutral and basic amino acids. After adsorption of the hydrolysate (5 litres) on the large column, and washing with water until the eluate was acid free, the amino acids were displaced with 75 mm-NaOH (80 litres). After 50 litres had run to waste 150 ml fractions were collected at a flow rate of 8 ml/min until the alkali was eluted from the last column. The fractions were analysed as described below.

Stage 2: anion-exchange displacement chromatography. Resolution of the basic amino acids was further improved by separation of the basic fractions from the cation-exchange columns on a series of anion-exchange columns. This technique has the advantage that the order of displacement of the amino acids from these columns is the reverse of that from acidic columns, thus decreasing the contamination from overlapping bands. Three columns of diminishing diameter, 2.5 cm \times 25 cm, 1.72 cm \times 16 cm and 1.2 cm \times 21 cm, were filled with Dowex 2 (8% divinylbenzene) converted from the Cl^- form into the OH^- form by elution with 2 M-NaOH. After the column had been washed with water the samples were applied to the largest

column and eluted with 0.1M-HCl. All solutions were boiled before use to remove CO₂ and prevent gassing on the columns.

Stage 3: elution chromatography with volatile buffers. The Technicon AutoAnalyzer was adapted for the use of volatile buffers based on the system described by Padieu, Malekina & Schapira (1965). A column (1.2 cm diam. × 60 cm) was filled with Zeo-Karb 225 (30–40 μm bead size) separated by the technique described by Hamilton (1958). The column was maintained at 60°C and the amino acids were eluted with a gradient formed by running 1.0M-pyridine-formic acid buffer, pH 5.0, into the mixing vessel containing 350 ml of 0.1M-pyridine-formic acid buffer, pH 2.9. A split-stream device was attached to the end of the column and one-twentieth of the eluate pumped through the modified ninhydrin system (Padieu *et al.* 1965), water being used to make up the volume. The remainder of the eluate was collected with an automatic fraction collector. The flow rate was adjusted to 40 ml/h and 10 min fractions were collected. The column was regenerated with 2M-pyridine and then starting buffer.

Stage 4: isolation from the basic column of the Beckman Amino Acid Analyzer. The basic column of the Beckman 120C Amino Acid Analyzer was lengthened to 60 cm to achieve resolution of the basic reduced cross-link components from hydroxylysine, lysine and ammonia. The standard 0.35M-sodium citrate buffer, pH 5.28, was employed. As a final isolation stage the radioactive components isolated from the Technicon volatile-buffer system were eluted from the extended basic column. The fractions under the specific peak required were combined and desalted by adsorption on a short column (5 cm × 0.5 cm) of Amberlite GC-120 washed with water and displaced by 2M-NH₃. The NH₃ was then removed by evaporation *in vacuo*.

Analysis of column fractions. The fractions collected from the displacement and elution chromatography columns were analysed for radioactivity by dissolving a portion (0.2 ml) in 3 ml of Bray's (1960) solution and measuring the ³H and ¹⁴C radioactivity with a Packard 3375 liquid-scintillation counter. Amino acids were identified by paper chromatography against standard amino acids with the single-phase system butan-1-ol-acetic acid-water (4:1:1, by vol.). Radioactivity of the separated components on paper chromatograms or paper electrophoresis was measured by cutting the paper strip into 1 cm squares, immersing the paper in the scintillation liquid [0.5% 2,5-diphenyloxazole (PPO) + 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) in toluene] and counting in the Packard 3375 instrument.

High-voltage paper electrophoresis. Electrophoresis was carried out on Whatman no. 1 paper strip in 0.1% ammonium carbonate buffer, pH 8.7, at 50 V/cm for 30 min in a flat-bed type of apparatus (AB Analysteknik, Vellentuna, Sweden) cooled by cold-water sprays. Mobilities were corrected for endosmotic flow by observation of the movement of glucose applied to both sides of the starting line. Amino acids were developed with ninhydrin, and glucose in the side strips with aniline phthalate.

G.l.c. Measurements were made by using a Pye series 104 chromatograph, with a 9 ft glass column. The stationary phase was GE XE-60 (Applied Science Laboratories Inc., State College, Pa., U.S.A.)—FD 1265 fluid (Dow Corning Corp., Midland, Mich., U.S.A.)—MS 200 Silicene

fluid M.F.C. (Hopkin and Williams Ltd.) (46:27:27, by wt.). The column was packed with deactivated Diatoport S (80–100 mesh) coated with 2.5% (w/v) of mixed stationary phase, and conditioned at 250°C overnight (Darbre & Islam, 1968).

T.l.c. Plates were made from Silica gel G (E. Merck A.-G., Darmstadt, Germany). The plates used were on microscope slides, prepared by using a suspension of silica gel in chloroform. The solvent system used was butan-1-ol-acetic acid-water (12:3:5, by vol.). The thin-layer plates were developed either by spraying with ninhydrin or 2M-H₂SO₄ with subsequent heating. Preparative t.l.c. was carried out by the method described by Halpaap (1963).

Periodate oxidation and borohydride reduction. A 1–3 mol portion of the compound was dissolved in 2 ml of 0.2M-sodium phosphate buffer, pH 6.3, and 1 ml of 0.02M-NaIO₄ added. The reaction was allowed to proceed for 2 h at room temperature and the excess of periodate was removed by the addition of ethylene glycol. NaB³H₄ was added to reduce the aldehydes and the excess destroyed after 10 min by acidification with acetic acid. The mixture was desalted on Amberlite GC-120 before analysis on the Technicon AutoAnalyzer.

Elemental analysis. Carbon, hydrogen and nitrogen analyses were carried out with a Hewlett Packard 185 C, H and N Analyzer, incorporating a Cahn Ratio electro-balance.

Mass spectrometry. All mass spectra were recorded on an L.K.B. 9000 GC-MS instrument with direct insertion of the sample into the ion source. Accurate mass ions were obtained by using the peak matcher against a known standard, generally perfluorokerosene.

Preparation of trifluoroacetyl methyl esters. A portion of material (1 mg) was added to anhydrous redistilled methanol (2 ml) and dry HCl was bubbled slowly through the solution for 35 min at room temperature. Complete reaction was checked by using t.l.c. Evaporation to dryness *in vacuo* afforded a gum, which was treated with trifluoroacetic anhydride (0.2–0.5 ml, depending on the number of reactive groups present) for 3 h at room temperature. T.l.c. indicated the absence of starting material. Evaporation to dryness *in vacuo* gave the required volatile derivative.

Trifluoroacetyl ethyl esters. The ethyl ester was prepared from the methyl ester by bubbling HCl through an ethanol solution as above, but the reaction mixture was then kept at room temperature for a further 2 h before evaporation of the solvent *in vacuo*. Treatment with trifluoroacetic anhydride, as above, gave the trifluoroacetyl ethyl ester.

Chemical synthesis of the proposed reduced cross-link and precursor

Synthesis of hydroxynorleucine. Hydroxynorleucine, the reduced form of the lysine-derived aldehyde, α-amino-adipic δ-semialdehyde, was synthesized by the method of Gaudry (1948). The product obtained was recrystallized from water, m.p. 238–239°C (Found: C, 48.5; H, 8.8; N, 8.8. Calc. for C₆H₁₃NO₃: C, 49.0; N, 9.5%).

Synthesis of hydroxylysionorleucine. The procedure was based on the scheme presented by Franzblau, Faris & Papaioannou (1969) for the synthesis of lysionorleucine based on that of Speck, Rowley & Horecker (1963) for the

synthesis of glyceryl-lysine. The preparation involved a coupling reaction between 5- δ -bromobutylhydantoin and the ϵ -amino group of hydroxylysine (Scheme 1). The reactants were prepared as follows.

(i) Hydroxylysine methyl ester. DL-5-Hydroxylysine hydrochloride (50 mg) was added to dry redistilled methanol (5 ml) and anhydrous HCl gas bubbled slowly through the reaction mixture for 40 min. The solution was evaporated to dryness *in vacuo*, the temperature being kept below 30°C, to give a gum, which resisted crystallization. T.l.c. showed the presence of only one compound, which was not hydroxylysine.

(ii) N^α -Trifluoroacetylhydroxylysine methyl ester (Weygand & Geiger, 1956). Hydroxylysine methyl ester (prepared as described above) was dissolved in trifluoroacetic acid (0.4 ml) and the solution cooled in ice-water; trifluoroacetic anhydride (0.06 ml) was added and the reaction stirred vigorously for 15 min. The cooled solution was evaporated to dryness *in vacuo* at 0°C. T.l.c. of the gummy residue showed that the product was mainly the N^α -trifluoroacetyl derivative (ninhydrin-positive), together with a little unchanged starting material (ninhydrin-positive) and the $N^\epsilon N^\alpha$ -ditrifluoroacetyl derivative (detection with 2M-H₂SO₄ and heating). Purification of the reaction mixture was not attempted before coupling with 5- δ -bromobutylhydantoin.

(iii) 5- δ -Bromobutylhydantoin. This was synthesized by a slight modification of the procedure detailed by Gaudry (1948). 5- δ -Hydroxybutylhydantoin was first synthesized from 2,3-dihydropyran. The product was recrystallized from water, m.p. 149–150°C (lit. m.p. 152–153°C) (Found: C, 48.8; H, 7.2; N, 16.3. Calc. for C₇H₁₂N₂O₃: C, 48.8; H, 7.0; N, 16.3%). The 5- δ -hydroxybutylhydantoin (1.2 g) was then treated with 45% (w/v) HBr solution in acetic acid (15 ml) for 24 h at room temperature. Evaporation *in vacuo* gave a dark-brown gum,

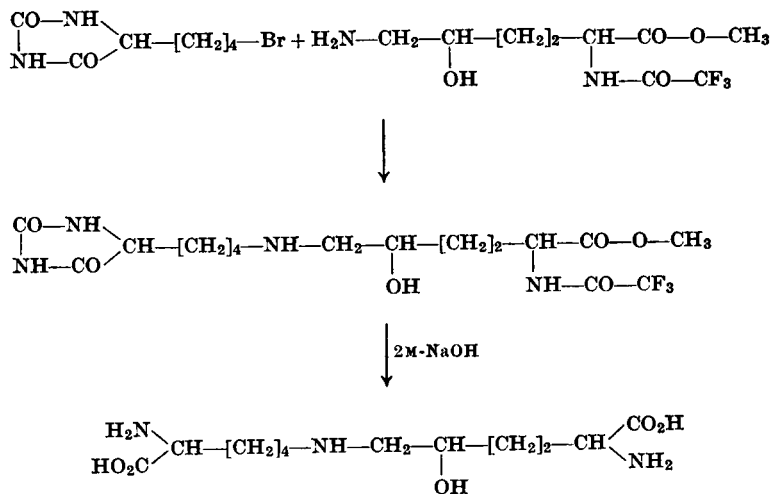
which crystallized from water. Repeated crystallization gave a white solid, m.p. 128–129°C (lit. m.p. 129–130°C). T.l.c. showed the presence of only one compound (Found: C, 35.7; H, 4.7; N, 11.4. Calc. for C₇H₁₁BrN₂O₂: C, 35.7; H, 4.7; N, 11.9%).

(iv) Hydroxylysinonorleucine. The reaction product from the trifluoroacetylation of hydroxylysine was dissolved in tetrahydrofuran (5 ml), and 5- δ -bromobutylhydantoin (44 mg) was added, together with triethylamine (0.05 ml). The reaction was refluxed for 24 h, cooled and filtered. The filtrate was concentrated under vacuum and the resulting thick syrup treated with 2M-NaOH (2 ml) in a sealed vial at 105°C for 16 h. The hydrolysate was filtered and the material was purified by fractionation on an amino acid analyser. The material was too hygroscopic for elemental analysis.

RESULTS

Identification of cross-links by borohydride reduction

Reduction of the intact calf tendon collagen fibres with tritiated sodium borohydride and subsequent analysis with the Technicon AutoAnalyzer, with volatile buffers, revealed the presence of a number of radioactive peaks remote from the normal amino acids. The pattern was very similar to that of rat tail tendon with the addition of a number of new peaks (Fig. 1). The non-identity of these peaks with normal amino acids was confirmed by paper chromatography by using ninhydrin to detect the amino acids and the Packard scintillation counter to detect the radioactive components. It is essential to demonstrate that these reduced com-



N^ϵ -(5-Amino-5-carboxypentyl)hydroxylysine
(hydroxylysinonorleucine)

Scheme 1. Chemical synthesis of hydroxylysinonorleucine.

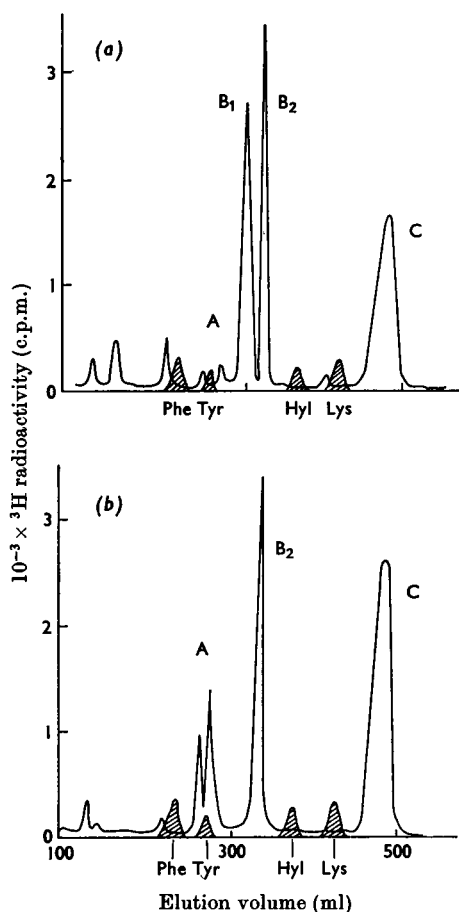


Fig. 1. Elution patterns of ^3H -labelled reducible components obtained from the acid hydrolysates of intact collagen fibres reduced with NaB^3H_4 . (a) Calf achilles tendon; (b) rat tail tendon. Hatched areas denote relative positions of normal amino acids; areas of peaks not to scale.

ponents were involved in the cross-linking process and were not artifacts produced during reduction. The following series of experiments was therefore carried out to determine the nature of the components before attempting to isolate them.

Analysis of lathyritic collagen

When leg tendons from embryonic chicks previously injected with β -aminopropionitrile were reduced and analysed under the same conditions the proportion of all the reducible components decreased (Fig. 2a), confirming the involvement of the material in these peaks in the cross-linking process.

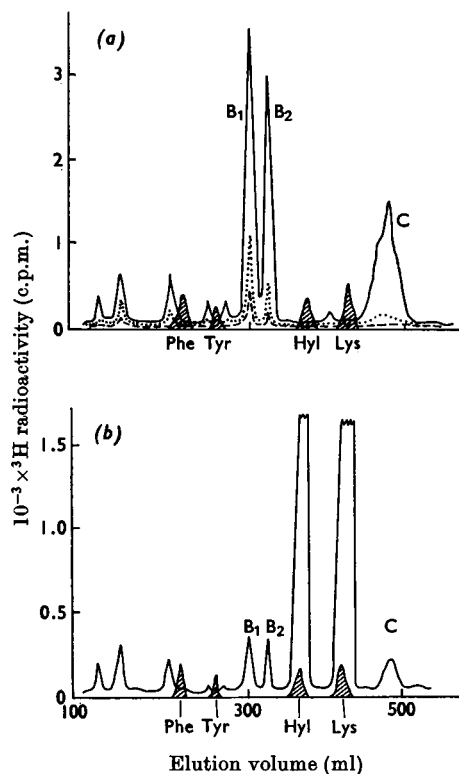


Fig. 2. Distribution of ^3H radioactivity of acid hydrolysates of reduced embryonic chick leg tendons. (a) Lathyritic collagen reduced with NaB^3H_4 : —, control; 15 mg of β -aminopropionitrile per egg; ----, 30 mg of β -aminopropionitrile per egg. (b) [^3H]Lysine-labelled embryonic chick tendon collagen reduced with non-radioactive borohydride. Hatched area denotes position of normal amino acids.

The inhibition of the biosynthesis of all the cross-links in experimental lathyrisms provides a good test for the involvement of a component in the cross-linking process.

Involvement of lysine

Analysis of fibres obtained from chicks previously injected with [^3H]- or [^{14}C]-lysine, then reduced with non-radioactive sodium borohydride, demonstrated that all the major peaks previously detected by tritiated borohydride were again radioactive (Fig. 2b), confirming that all the reducible components in collagen are derived from lysine. The known involvement of lysine in the intramolecular cross-link (Piez, 1968) lends further support to the contention that these radioactive components are involved in the cross-linking process.

Identification of type of cross-link corresponding to each radioactive peak

Intermolecular cross-links. Since the intramolecular cross-link and its precursor are reducible a number of experiments were carried out to demonstrate the nature of some of the radioactive peaks, e.g. whether they correspond to the cross-link precursor, i.e. reduced α -aminoadipic δ -semialdehyde, reduced intramolecular cross-links, or reduced intermolecular cross-links.

Comparison of the elution pattern obtained from intact calf collagen fibres (Fig. 1a) with that of reduced tropocollagen solution (Fig. 3a), i.e. the reduction of intramolecular cross-links and their precursors in the absence of intermolecular bonds, demonstrated the absence of the three major peaks B₁, B₂ and C, suggesting the involvement of these components in the intermolecular cross-linking (Fig. 3).

Reduction of the fibrils produced by reprecipitation of the tropocollagen solution again revealed the presence of peaks B₂ and C (Fig. 3c), thus supporting the suggestion that these components act as intermolecular cross-links.

The elution patterns of fibres treated with D-penicillamine, and similar reagents, before borohydride reduction were compared with normal reduced tendon. The absence of peaks B₂ and C (Fig. 4) after penicillamine treatment further demonstrated that one or both components were involved in intermolecular cross-linking, since previous studies have demonstrated that penicillamine treatment decreases the tensile strength, presumably by breaking the intermolecular bonds (Bailey, 1968a). Peak B₁ was still present, suggesting that this component was relatively more stable.

Thermal denaturation of the fibres before reduction again resulted in the removal of peaks B₂ and C (Fig. 4). This treatment produced a more complex pattern, presumably due to the production of additional components during the rupture of the labile cross-links B₂ and C. Peak B₁ was still present, confirming its stability.

It was concluded that components B₁, B₂ and C were intermolecular bonds derived from lysine, B₁ being relatively stable to thermal denaturation, dilute acids and α -amino- β -thiols, whereas B₂ and C were extremely labile bonds.

Intramolecular cross-link and cross-link precursors.

Reduction of highly purified tropocollagen solution revealed the presence of a number of components, two major peaks eluting in the acidic region (D, E) and a further two peaks (A) eluting in the region of tyrosine (Fig. 3a). Reduction of thermally denatured collagen solution produced a similar pattern, indicating that these components were thermally stable. Reduction of α -1 chains previously

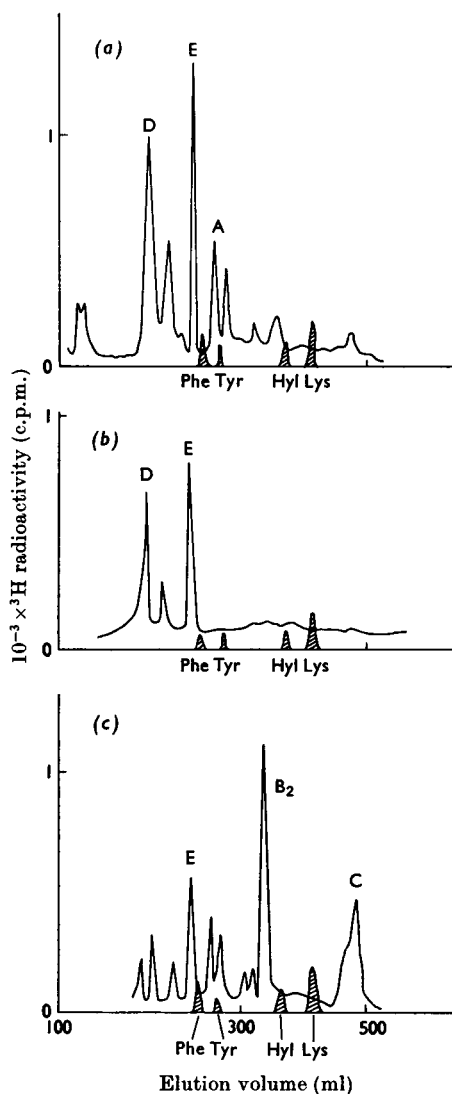


Fig. 3. Elution patterns of ³H-labelled reducible components obtained from the acid hydrolysates of collagen reduced with NaB³H₄. (a) Purified calf tendon tropocollagen solution (pH 7.0; 0.1M-calcium acetate); (b) α -1 components isolated from purified denatured tropocollagen (calf tendon) on CM-cellulose; (c) fibres reprecipitated by dialysis of purified calf tropocollagen solutions against 0.9% NaCl.

separated on CM-cellulose columns revealed only the two main radioactive peaks in the acidic region (D and E), whereas the peaks eluting near tyrosine in reduced tropocollagen were absent, suggesting that these peaks may be involved in intramolecular cross-links. The peaks present in reduced α -1

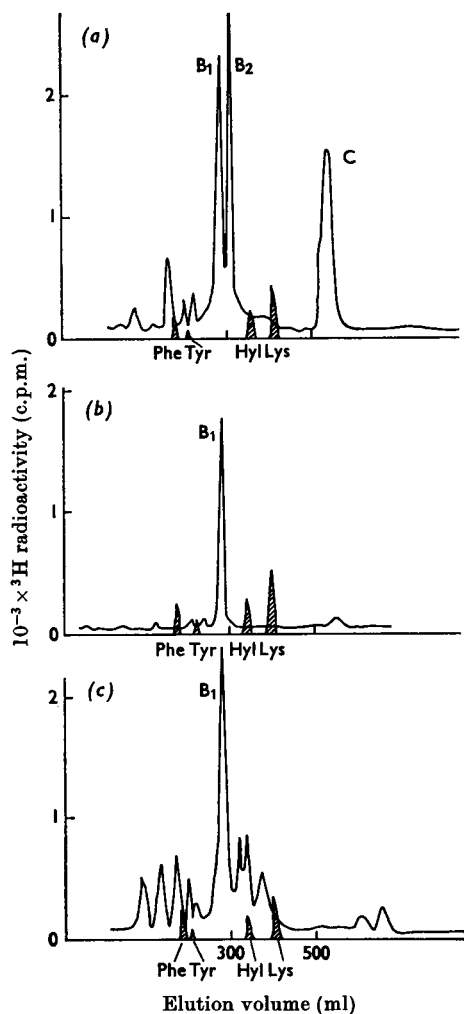


Fig. 4. Effect of D-penicillamine and thermal denaturation on intact collagen fibres from calf achilles tendon, demonstrated by comparison of the reducible components in the acid hydrolysates. (a) Normal calf tendon reduced with NaB^3H_4 ; (b) 0.1M-D-penicillamine-treated calf tendon (*in vitro*) reduced with NaB^3H_4 ; (c) thermally denatured (70°C for 2 min) calf tendon reduced with NaB^3H_4 .

chains (D and E) might then represent the cross-link precursors.

Isolation of reduced components

Fractions B_1 and B_2 . The first stage of isolation of fractions B_1 and B_2 was achieved on the cation-displacement column (Fig. 5). Preliminary examination of the fractions on the Technicon column revealed the presence of components B_1 and B_2 in

band 3. Fractions collected under band 3 were therefore combined and applied to the anion-displacement column (Fig. 6). Analysis of band C from the anion-displacement column on the Technicon column revealed both B_1 and B_2 components. The fractions in this band were combined, divided into four equal portions and chromatographed on the volatile buffer elution system to separate components B_1 and B_2 . To remove the final trace of contamination of the two peaks the fractions were finally isolated from the Beckman Amino Acid Analyzer owing to its better resolution. The compounds were then desalted before analysis.

Analysis of fraction B_2 . (a) Electrophoresis and g.l.c. Fraction B_2 gave a single ninhydrin-positive radioactive spot on paper electrophoresis. Comparison of its mobility, corrected for endosmotic flow, with lysine, lysinonorleucine and merodesmosine showed that component B_2 was very slightly less basic than lysinonorleucine. G.l.c. of the methyl ester of compound B_2 revealed the presence of two peaks, suggesting the possibility of isomers, since two peaks similar to the twin peaks of hydroxylysine had also been revealed on the Beckman Amino Acid Analyzer.

(b) Mass-spectral analyses. The most satisfactory volatile derivatives for g.l.c. and mass-spectral analyses were found to be the *NO*-trifluoroacetyl methyl and ethyl esters (Darbre & Islam, 1968). The component B_2 tetratrifluoroacetyl methyl ester gave a mass ion of 703 by direct injection into the ion source of the mass spectrometer at 140°C . An exact mass measurement, by peak matching against perfluorokerosene, afforded a mass ion of 703.14 ($\text{C}_{22}\text{H}_{25}\text{F}_{12}\text{N}_3\text{O}$, requires mol.wt. 703.14). The fragmentation pattern was consistent with the compound being N^ϵ -(5-amino-5-carboxypentyl)-hydroxylysine, or hydroxylysinonorleucine in the nomenclature adopted by Franzblau, Sinex, Faris & Lampidis (1965) for lysinonorleucine. Mass-spectral analysis of the two peaks of compound B_2 isolated from the Beckman Amino Acid Analyzer gave identical spectra establishing their identity as isomers. The presence of isomers may be expected from the proposed structure, since the molecule is derived from hydroxylysine, which exhibits diastereoisomerism. Epimerization at C-2 occurs during acid hydrolysis of hydroxylysine (Hamilton & Anderson, 1955).

(c) Structural studies on component B_2 by using periodate oxidation. On the basis of the proposed structure of component B_2 the compound could be expected to be cleaved by periodate, and after reduction of the reaction products with NaB^3H_4 yield radioactive lysine and hydroxynorvaline (Scheme 2). Oxidation of component B_2 and reduction with non-radioactive borohydride produced radioactive lysine as shown by analysis on the

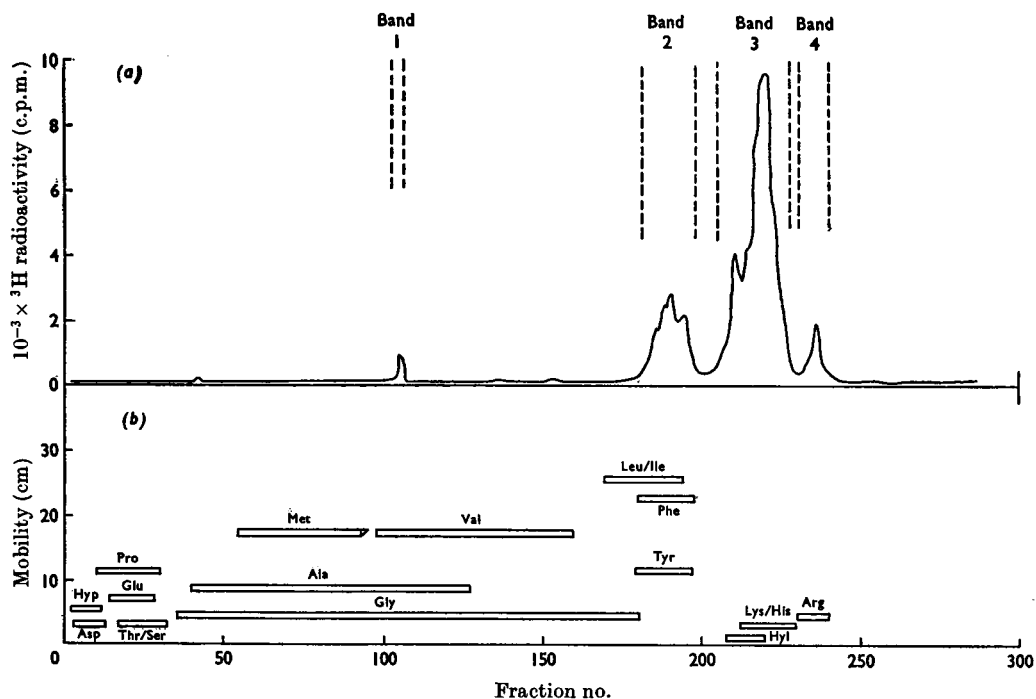


Fig. 5. Primary fractionation of the hydrolysis products of 200 g of NaB^3H_4 -reduced calf achilles tendon by cation-exchange displacement chromatography. (a) Distribution of the radioactive components; (b) identification of the amino acids by paper chromatography with respect to their position of displacement from the column.

Technicon AutoAnalyzer, the Beckman Amino Acid Analyzer and by electrophoresis. Reduction with tritiated sodium borohydride revealed the presence of two additional peaks eluted near glycine on the AutoAnalyzer. Comparison of the chromatographic properties of the first peak with authentic hydroxynorvaline, synthesized by periodate oxidation of N^α -trifluoroacetylhydroxylysine, confirmed the identity of this component as hydroxynorvaline. The second radioactive peak, which occurred on oxidation and reduction of both component B_2 and hydroxylysine, was shown by chromatography to be $[4\text{-}^3\text{H}]$ proline, presumably formed by ring closure α -aminoglutaric acid of γ -semialdehyde.

(d) Confirmation of structure by chemical synthesis. On the basis of the proposed formula a chemical synthesis of the compound was designed to confirm the structure. The synthetic hydroxylisinonorleucine eluted from the Beckman Amino Acid Analyzer as twin peaks and co-chromatographed with the isolated component B_2 . The electrophoretic mobility and the mass spectra of the compound were identical with those of the

isolated component B_2 , thus confirming the latter's identity as hydroxylisinonorleucine.

Analysis of fraction B_1 . The incorporation of $[^{14}\text{C}]$ lysine into component B_1 indicated that the compound was derived from either lysine or hydroxylysine. The electrophoretic mobility and its elution pattern from the Amino Acid Analyzer suggested that it was less basic than hydroxylisinonorleucine. This was also consistent with the lower nitrogen analysis.

(a) Mass spectra. The trifluoroacetyl methyl ester of component B_1 on direct insertion into the ion source at 110°C gave an exact mass ion of 816.10 when peak-matched against perfluorokerosene ($\text{C}_{24}\text{H}_{23}\text{F}_{15}\text{N}_2\text{O}_{12}$ requires mol.wt. 816.10). The trifluoroacetyl ethyl ester gave a mass ion of 844; the difference between this mass and that of the methyl ester indicated the presence of two carboxyl groups. The mass ion obtained, together with the interpretation of the fragmentation pattern, was consistent with the component B_1 being the reduced form of the aldol derived from the reaction of the δ -semialdehyde of lysine and hydroxylysine, two isomers of which exist (I and II).

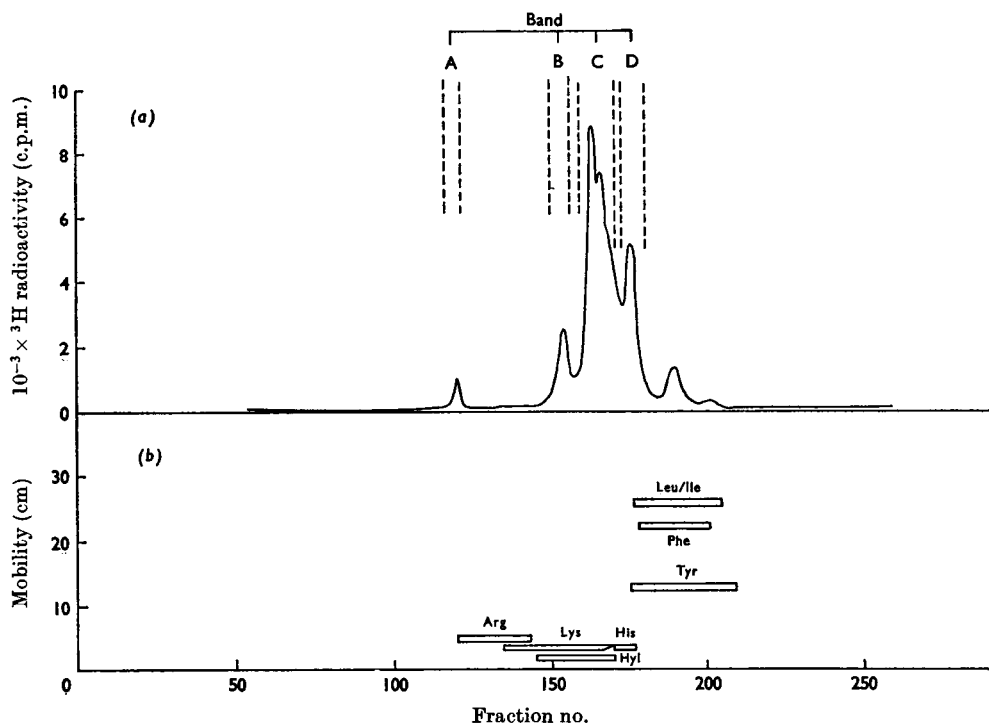
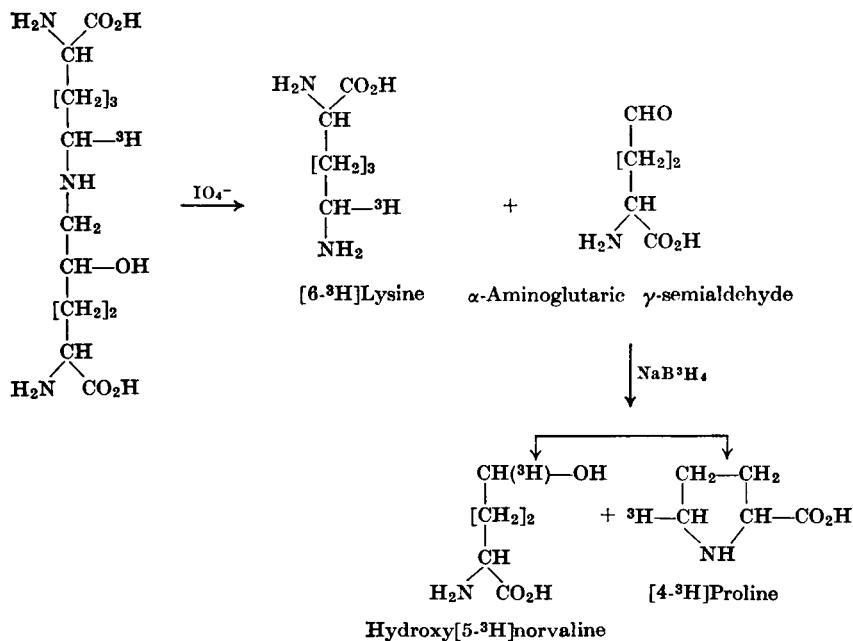
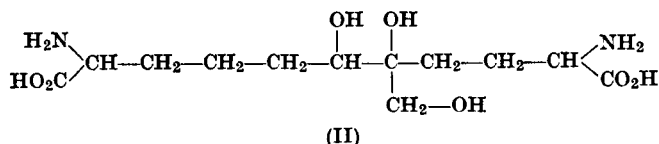
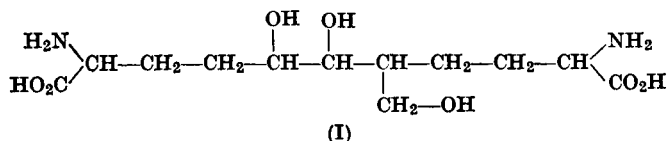


Fig. 6. Secondary fractionation of band 3 from the cation-exchange displacement columns on a series of anion displacement columns. (a) Displacement position of the NaB^3H_4 -reducible components; (b) relative positions of the normal amino acids.



Scheme 2. Periodate cleavage of component B₂.



(b) Periodate oxidation. Periodate oxidation of component B₁ followed by borohydride reduction and analysis on the Technicon AutoAnalyzer revealed the formation of hydroxynorvaline and two unknown peaks. Insufficient material was available for confirmation of the identity of hydroxynorvaline by isolation. Hydroxynorvaline would be produced from both isomers, but isomer (II) would produce a mixture of hydroxynorvaline and hydroxynorleucine. In view of the absence of the latter, isomer (I) might be the preferred configuration.

Analysis of fractions D and E. The peaks eluted before glycine (D) and immediately before phenylalanine (E) from the volatile buffer system were present in reduced tropocollagen, reduced gelatin, reduced α -1 chain and reduced telopeptide (Fig. 3). Isolation of component D was attempted from the reduced telopeptides, since the contamination due to glycine and alanine was greatly decreased. This fraction also contained component E (Fig. 3). The material isolated from the volatile buffer system was further purified by t.l.c. on silica gel plates and finally on the Beckman Amino Acid Analyzer. The mass spectra of the volatile derivative of the isolated compound identified it as ϵ -hydroxynorleucine. This compound was therefore synthesized by the method of Gaudry (1948); the isolated compound D co-chromatographed with the synthesized material, and the mass spectra were identical. Chlorination of the hydroxyl group was found to occur during formation of the methyl ester. The derivative used therefore, was di-*ON*-trifluoroacetylhydroxynorleucine, with an unprotected carboxyl group. Insertion of the sample, by direct probe, into the ion source at 40°C gave an exact (mass+1) ion of 340.06, with C₂₃H₄₈ as the mass standard (C₁₀H₁₁F₆NO₃ requires mol.wt. 339.05). The synthesized hydroxynorleucine gave a (mass+1) ion of 340 and an identical fragmentation pattern.

Gallop, Blumenfeld, Henson & Schneider (1968) have reported that acid hydrolysis of ϵ -hydroxynorleucine results in the formation of ϵ -chloronorleucine. Treatment of the synthesized ϵ -hydroxynorleucine with 6*M*-hydrochloric acid (105°C for

12h) produced a 55% conversion into the ϵ -chloronorleucine. The ϵ -chloronorleucine co-chromatographed with the peak eluted before phenylalanine (E). Confirmation that component E was ϵ -chloronorleucine was obtained from the mass spectrum of the *N*-trifluoroacetyl methyl ester. Direct injection of the sample into the ion source at 70°C gave a (mass+1) ion of 276.06, when peak-matched, with C₁₉H₄₀ (C₉H₁₃ClF₃NO₃ requires mol.wt. 275.05). Its structure as ϵ -chloronorleucine was supported by the fragmentation pattern, which showed peaks at both 276 and 278, the former being three times as intense as the latter, indicating the presence of chlorine in its isotopic forms of atomic weights 35 and 37, present in a 3:1 ratio.

DISCUSSION

Identification of the reducible components. The analysis of the hydrolysis products of mild reduction of collagen fibres from calf achilles tendon revealed the presence of about eight major components. Their absence on reduction of lathyrin collagen and their derivation from lysine indicated their probable involvement in the general biosynthesis of the cross-links, as either precursors, intermediates or permanent cross-links. Comparative studies with highly purified reduced reprecipitated fibrils, tropocollagen, and isolated α -chains suggested that the three major components in the basic region, B₁, B₂ and C (Fig. 1), were involved in the intermolecular cross-linking.

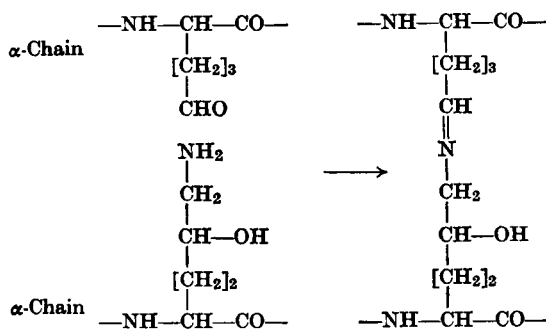
Identification by mass spectrometry of component B₂ as *N* ϵ -(5-amino-5-carboxypentyl)hydroxyllysine confirmed the nature of this component as a possible cross-linking agent. Since this compound appears in the acid hydrolysate after reduction it must be concluded that it exists in the collagen fibre in the aldimine-bond form, i.e. 6,7-dehydrohydroxyllysino-norleucine, and such a cross-link could be formed biosynthetically through the condensation of the carbonyl function of the lysine-derived aldehyde on one peptide chain with the

ϵ -amino group of hydroxylysine on an adjacent peptide chain (Scheme 3).

It might have been expected that the aldimine bond involved lysine rather than, or in addition to, hydroxylysine to give lysinonorleucine on reduction. Although it is present in elastin (Franzblau *et al.* 1965) we have been unable to detect this compound in collagen.

The second intermolecular component (B_1) was found to be relatively more stable to α -amino- β -thiols, thermal denaturation and dilute acids. This new amino acid was partially characterized by mass spectrometry as the aldol resulting from the reaction of the lysine-derived aldehyde (allysine) and that of hydroxylysine (hydroxyallysine). The reaction of these aldehydes could result in the formation of two isomeric forms of the aldol (Scheme 4), related to the reduced forms (I) and (II). It is not at present possible to distinguish which isomer predominates.

Although no formal chemical proof of the structural configuration is as yet available, the structures proposed are the most reasonable based on the present evidence. We intend to refer to this cross-link by the non-systematic name 'syndesine' (Greek *συνδέω*, meaning bind together).



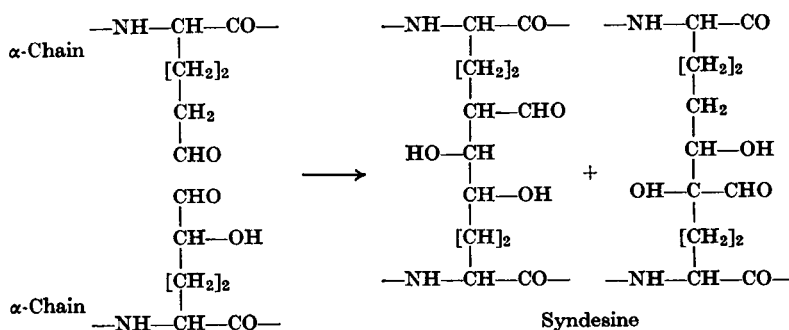
Scheme 3.

Support for these proposals arises from the isolation of hydroxynorleucine, the reduction product of one of the cross-link precursors, allysine. The corresponding reduction product from the second cross-link precursor, hydroxyallysine, has been tentatively identified chromatographically, but insufficient material is as yet available to confirm this.

The identification of the third intermolecular component (C) has not yet been completed, but preliminary studies suggest that it is a mixture of components of higher molecular weight and is probably derived from the reduction of an aldimine bond.

Nature of the intermolecular bonds. Two of the proposed intermolecular bonds, dehydrohydroxy-lysinonorleucine and the non-reduced form of component C, are extremely labile, being readily cleaved by treatment with α -amino- β -thiols, dilute acids and thermal denaturation. The third cross-link syndesine, however, appeared to be relatively more stable under these conditions. The inability of dilute acids to rupture this bond is demonstrated by the absence of syndesine on reduction of fibrils reprecipitated from purified tropocollagen solution on dialysis against iso-osmotic saline. In contrast, the aldimine bonds (non-reduced forms of B_2 and C) are ruptured during extraction and re-form on reprecipitation. Thus, although the aldimine-bond type of cross-links may confer stability on the fibre in the physiological state, they cannot account for the general insolubility of collagen fibres or the presence of cross-linked components detectable after gelatinization. The syndesine cross-link, however, may account, at least in part, for the insolubility. It is significant that young rat tail tendon does not possess this cross-link (Fig. 1) and is almost completely soluble in dilute acids. In contrast, bone collagen is virtually insoluble and contains predominantly the syndesine cross-link (Bailey, Fowler & Peach, 1969).

The presence of these labile aldimine bonds accounts for the ability of α -amino- β -thiols, such as *D*-penicillamine, to render collagenous tissue fragile



Scheme 4.

(Harkness & Harkness, 1966; Nimni, 1966). These compounds can cleave the pre-existing aldimine cross-links (Bailey, 1968a), and inhibit further cross-link formation presumably by forming a thiazolidene complex (Heyl, Harris & Folkers, 1948) with the lysine aldehyde. It is significant that this effect is most dramatic with young rat tissue, which contains little or no syndesine, in comparison with other mammalian tissue, which contain this additional more stable cross-link. It is notable too that the cuticle of *Ascaris lumbricoides* contains the only collagen lacking hydroxylysine, presumably owing to the lack of molecular oxygen for hydroxylation in its anaerobic environment (Fujimoto & Tamiya, 1962), and is stabilized by a completely different cross-linking system involving disulphide bonds (McBride & Harrington, 1967).

From the results obtained with embryonic chick leg tendon from eggs previously injected with labelled lysine, it can be estimated that young collagen contained one syndesine cross-link and one hydroxylysinonorleucine cross-link for every two tropocollagen molecules. This suggests that these bonds play a major role in the stabilization of the collagen fibre. The complete absence of cross-links from lathyrin collagen, the apparently specific involvement of only lysine derivatives in this process and the absence of other significant components involving lysine suggests it is unnecessary to invoke the participation of other types of cross-links. However, it is highly probable that these cross-links are intermediates in the overall biosynthesis of interchain cross-links, since there is a marked decrease in the proportion of reducible components with increase in age after maturity (Bailey, 1969). It may be that these reducible components are finally stabilized in a non-reducible form.

Location of the cross-links. The lysine-derived aldehyde, which appears to be the fundamental precursor for the cross-linking process in collagen, has been shown to reside solely in the telopeptide region of rat skin tropocollagen (Bornstein & Piez, 1966). Its presence in the non-helical region affords a ready explanation for the enzymic oxidative deamination of a single specific lysine residue in the molecule. We have isolated the reduced form of this aldehyde from the telopeptide cleaved from the molecules by pepsin digestion. Further, reduction of fibrils formed from tropocollagen after cleavage of the telopeptide resulted in the virtual absence of the reducible components. The *N*-terminal telopeptide region does not contain hydroxylysine (Kang, Bornstein & Piez, 1967), clearly indicating that hydroxylysinonorleucine must exist as an intermolecular bond from the telopeptide region of one molecule to the body of the second molecule. This would be entirely consistent with the generally

accepted 'quarter-stagger' alignment of the molecules in the native fibril.

The formation of syndesine from the hydroxylysine-derived aldehyde suggests that hydroxylysine must also occur in the non-helical regions accessible to the amine oxidase. Indeed Miller, Lane & Piez (1969) have reported that the lysine at position 9 in the telopeptide of bone collagen is 50% hydroxylated. Thus in bone collagen both lysine and hydroxylysine are present in a region accessible to the amine oxidase. It may be that in bovine tendon collagen a lower proportion of the lysine is hydroxylated, and this would be consistent with the decreased content of syndesine in calf tendon compared with bone. However, if these aldehydes occur only in the *N*-terminal telopeptide region the resulting intermolecular bonding would entail a side-to-side aggregation of the tropocollagen molecules with their *N*-terminal ends in register. On the 'quarter-stagger' hypothesis only a small proportion of the molecules would have their ends aligned in this manner and the cross-link would be of minor importance. An alternative hypothesis, assuming the 'quarter-stagger' alignment to be correct, is that the lysine or hydroxylysine aldehydes also occur at some point along the body of the molecule. Detailed sequence studies on the peptide chain have only been carried out on rat skin, but this tissue does not contain the syndesine cross-link. Sequence studies on bovine tendon should resolve the possibility of additional aldehydes along the body of the molecule, either on an extra non-helical peptide or within the main peptide chain. Actually it may not be necessary to have an additional non-helical peptide along the triple helix for the sake of enzyme accessibility, since the ability of enzymes to react with specific hydroxylysines within the triple helix is evident from the location of the Glu-Gal-Hyl residue some 300 Å from the *N*-terminal end of the molecule (Piez, 1970). The studies of Kuhn, Fietzek & Kuhn (1966) are pertinent, since they suggest that a side-to-side cross-link is present in bovine tendon and virtually absent from rat tail or rat skin collagen. It is possible that syndesine may be related to this side-to-side cross-link.

Although far from complete, amino acid sequence studies on different collagens strongly suggest that the primary structures are virtually identical. It is therefore unlikely that differentiation into the various types of tissue is directly controlled by genes, but is in all probability due to the variations of the modifications after the primary chain synthesis. These modifications involve hydroxylation of proline and lysine, addition of carbohydrate moieties, and enzymic oxidation of specific lysine and hydroxylysine residues and the subsequent production of cross-links. Variations in these additional modifications would effect molecular inter-

actions between collagen molecules themselves and also interactions with other components of a particular tissue.

As far as mature collagens are concerned we have here demonstrated differences in the nature and extent of the reducible cross-links between rat tail tendon and bovine achilles tendon and within a species between bovine tendon, bone and cartilage. Further differences will become apparent as other tissues are examined and perhaps ultimately permit a correlation between structure and function.

These differences suggest that the variations are due to the molecules themselves rather than to outside influences. Therefore one may speculate that the involvement of hydroxylysine in the cross-links could be a factor controlling the extent of cross-linking in different tissues. The greater the proportion of the specific lysine hydroxylated, the more hydroxylysine available in an accessible position for enzymic conversion into the aldehyde and subsequent cross-link formation. If indeed this is the case future studies should reveal a smaller proportion of the telopeptide lysine hydroxylated in calf tendon collagen compared with the 50% in chick bone collagen.

The relationship to the cross-linking mechanism, if any, of the ability of certain hydroxylysines to attach disaccharide units of galactose and glucose is not at present clear. The possibility of the Gal-Glu unit sterically hindering the involvement of this specific hydroxylysine from cross-link formation cannot be overlooked, particularly in view of its position on the tropocollagen molecule, i.e. at the end of the overlap region.

From the present studies it is clear that collagen fibres contain both labile and more stable intermolecular cross-links. The labile cross-links are aldimine bonds whereas the more stable cross-links appear to be aldols, both types of bond being derived from lysine and hydroxylysine. It is believed that these cross-links play a major role in the stabilization of the fibre.

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