Polymeric C-Terminal Cross-Linked Material from Type-I Collagen

A MODIFIED METHOD FOR PURIFICATION, ANOMALOUS BEHAVIOUR ON GEL FILTRATION, MOLECULAR WEIGHT ESTIMATION, CARBOHYDRATE CONTENT AND LIPID CONTENT

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Polymeric cross-linked C-terminal peptide material (poly- α 1CB6) from mature bovine tendon type-I collagen was prepared and purified by a modification of the method previously described [Light & Bailey (1980) *Biochem. J.* **185**, 373–381]. Poly- α 1CB6 was shown to exhibit concentration-dependent aggregation effects on gel filtration due to interaction with the filtration medium. The material had an amino acid content that was very similar to a mixture of α 1CB6 and α 1CB5. The material was shown to be polydisperse with a mol.wt. range of 50 000–350 000, but chromatographic fractions were relatively homogeneous over this molecular weight range with respect to amino-acid composition. The heterogeneity of the material was not due to incomplete CNBr peptide cleavage, as poly- α 1CB6 did not contain detectable quantities of methionine. The material showed no discrete bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis but gave a constant blue stain throughout the molecular weight range described above. Lipid analysis showed that the partially purified material contained elevated levels of stearate when compared to the crude CNBr-digested starting material. This may indicate the specific association of a stearic-acid-rich lipid with the peptide material. On carbohydrate analysis poly- α 1CB6 was shown to contain only galactose and glucose at levels of 0.72 and 0.28% respectively. The carbohydrate and amino acid analyses indicated that $(\alpha$ 1CB6)₂– $(\alpha$ 1CB5)₁ may be the basic cross-linked structural unit of poly- α 1CB6. Oligomers of poly- α 1CB6 therefore may contain from one to eight $(\alpha$ 1CB6)₂– $(\alpha$ 1CB5)₁ units, although the carbohydrate analysis indicated that the higher molecular weight oligomers may be enriched in α 1CB6.

A fundamental requirement in the investigation and characterization of the so-called stable cross-link of mature collagen is an understanding of both the mechanism by which it arises and the regions of the collagen molecule involved in its formation. Much work has been carried out in the past with a view to determining the identity and location of the cross-links of newly formed collagen. The chemistry of the reducible cross-links is now well described [see Light & Bailey (1979a) for review] and the location of these cross-links is generally agreed to be in the N- and C-terminal non-helical regions (type I: Kang, 1972; Henkel *et al.*, 1976; type II: Miller & Robertson, 1973; type III: Nicholls & Bailey, 1980).

* Abbreviation used: SDS, sodium dodecyl sulphate.

Robins *et al.* (1973) showed that, during physiological maturation of skin and tendon, the numbers of reducible cross-links decreased to less than 10% of their values in newly formed connective tissue. It was speculated that these reducible cross-links were modified to a non-reducible and hence undetectable form by some unknown process that led to further stabilization of the collagen matrix. Evidence for this mechanism was elucidated independently by Davison (1978) and Light & Bailey (1979b). It was deduced that high-molecular-weight material observed in CNBr digests of mature type-I collagen from various sources was cross-linked by the stable bond (Light, 1979).

In a previous publication we showed (Light &

Bailey, 1980) that this cross-linked material could be isolated from CNBr digests of mature bovine tendon collagen in the form of a high-molecular-weight polymer of $\alpha 1CB6$, the $\alpha 1$ chain C-terminal peptide. This material, termed poly- $\alpha 1CB6$, consisted predominantly of $\alpha 1CB6$ and was stable to treatment by periodate.

In the present paper we report a modification to the original preparation procedure of poly- $\alpha 1CB6$ which allows the rapid processing of CNBr-digested tendon. Yields of up to 70% of the expected theoretical figures were obtained and the peptide was shown to be pure by SDS/polyacrylamide-gel electrophoresis. An investigation of the unusual gel filtration properties of poly- $\alpha 1CB6$ in solution is also presented, as well as its further characterization by molecular weight estimation, lipid and carbohydrate content.

Materials and Methods

Blue dextran, Sephadex and Sepharose products were obtained from Pharmacia, Uppsala, Sweden. Agarose A-1.5m and A-5m were obtained from BioRad, Bromley, Kent, U.K.

Gel filtration and velocity sedimentation studies were carried out using either 1M-CaCl₂/0.05M-Tris/HCl, pH 7.5 (buffer 1), or 2M-urea/0.15M-NaCl/0.02M-sodium phosphate, pH 7.5 (buffer 2).

Investigation of anomalous behaviour of poly- $\alpha 1CB6$ on gel filtration

Mature bovine Achilles tendon was prepared and digested with CNBr as previously described (Light & Bailey, 1979b). The digest was dissolved at a concentration of 50mg/ml in buffer 1 by incubating at 60°C for 30min. The solution was clarified by centrifugation for 30min at 40000g and 1ml was chromatographed on a column (40cm × 1.6cm) of A-1.5m agarose in buffer 1. The void volume peak (poly- $\alpha 1CB6$) was pooled and half of it was immediately re-chromatographed on the same A-1.5m agarose column under the same conditions. The other half of the void volume sample was simultaneously chromatographed on a column (60cm × 1.0cm) of Sepharose 4B equilibrated in buffer 1.

The behaviour of poly- $\alpha 1CB6$ on gel filtration on A-1.5m agarose was also investigated at high and low pH and in different buffer systems. In the first experiment the yield and distribution of poly- $\alpha 1CB6$ was observed after gel filtration in 1M-CaCl₂ on A-1.5m agarose at pH 3.0 (adjusted with 0.5M-acetic acid) and at pH 9.5 (buffered with 0.05M-glycine/NaOH buffer). The mean pore size of the agarose at these two pH values was calculated from the observed elution volume of bovine serum albumin by the method of Ackers (1964).

The chromatographic behaviour of poly- $\alpha 1CB6$ on A-1.5m agarose was also studied in 1M-NaCl and in 4M-urea. Both solutions were buffered at pH 7.5 with 0.05M-Tris/HCl.

Finally, the influence of protein concentration on the elution pattern of poly- $\alpha 1CB6$ from the A-1.5m column was investigated. The CNBr digest of mature bovine tendon dissolved at 5, 10, 25, 50 and 100mg/ml in buffer 1 was resolved on the A-1.5m column and the distribution and yield of poly- $\alpha 1CB6$ was estimated.

Bulk preparation and purification of poly- $\alpha 1CB6$

In general, for bulk preparations, 10g of CNBr-digested tendon was dissolved in buffer 1 to a final concentration of 50mg/ml by incubation at 60°C for 30min. The solution was clarified by centrifugation at 40000g for 30min at 20°C. The supernatant was collected and loaded on a column of A-1.5m agarose equilibrated in the same buffer. The column consisted of a glass cylinder 16cm in diameter and 14.5cm in length having a conical base of height 5cm. A buffer head was maintained at the open top of the column by means of a regulated drip feed from a reservoir. Flow rates of over 1000ml/h were obtained. Fractions (20ml) were collected and scanned at 280 and 230nm. Void volume material (poly- $\alpha 1CB6$) was pooled, dialysed exhaustively against distilled water and freeze-dried.

Partially purified poly- $\alpha 1CB6$ was redissolved in 2M-urea/0.2M-NaCl/0.02M-sodium phosphate, pH 7.5, and acidic protein contaminants were removed by passage through DEAE-Sepharose CL-6B (Light & Bailey, 1980). The material was again dialysed and freeze-dried before dissolving it in buffer 1 at 60°C for 10min. Some of the material (1g) at a concentration of 50mg/ml was loaded onto a column (55cm × 5cm) of Sepharose 4B equilibrated in the same buffer. Fractions were scanned at 230nm, pooled, dialysed against water and freeze-dried.

Molecular weight estimation of poly- $\alpha 1CB6$

The behaviour of collagen and collagen-derived peptides on gel filtration and SDS/polyacrylamide-gel electrophoresis is known to be anomalous with respect to molecular weight (Furthmayr & Timpl, 1971). For this reason, three methods were adopted to ascertain the molecular weight range of the poly- $\alpha 1CB6$ species.

(a) *SDS/polyacrylamide-gel electrophoresis.* Poly- $\alpha 1CB6$ was dissolved in a solution of 5% (w/v) SDS in 0.125M-Tris/HCl, pH 6.8, containing 10% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue at 60°C for 30min to give a final protein concentration of 5mg/ml. A sample (250µg of protein) was loaded onto each track of a 2mm-thick slab gel and electrophoresis was carried out at 200V at 4°C.

Solutions containing γ , β and α components of type-I collagen as well as type-I collagen CNBr peptides were run as molecular weight markers in tracks adjacent to poly- α 1CB6 samples. Collagen and collagen peptides were prepared as previously described (Light & Bailey, 1979b).

Electrophoresis was carried out in 5% (w/v) and 10% (w/v) polyacrylamide-slab gels polymerized in 0.375 M-Tris/HCl, pH 8.8, containing 0.1% SDS (Laemmli, 1970) and also in 3–10% (w/v) polyacrylamide gradient slab gels polymerized in the same buffer. Molecular weight estimations were made by plotting migration distances (band front) as R_F values (with respect to dye front) against the logarithm of molecular weight.

Gels were routinely stained, destained, dried and scanned in a Joyce-Loebl Chromoscan as previously described (Light & Bailey, 1979b).

(b) *Gel filtration.* Poly- α 1CB6 (5 mg/ml in buffer 1) was chromatographed on a column (62 cm $\times$ 1 cm) of Sepharose 4B equilibrated in buffer 1. The column effluent was monitored at 230 nm and 1 ml fractions were collected at a flow rate of 12.6 ml/h. Molecular weight limits for poly- α 1CB6 were estimated from a standard curve of elution volumes versus the logarithms of the molecular weights of the γ , β and α chains of type-I collagen and α_2 CB3,5, the α_2 -chain C-terminal CNBr peptide.

(c) *Sedimentation velocity in the analytical ultracentrifuge.* The sedimentation velocity of the poly- α 1CB6 species was determined at 22°C in an MSE Centriscan 75 using Schlieren optics. The protein was dissolved at a concentration of 3 mg/ml in both buffer 1 and buffer 2 and any undissolved solid was removed by centrifugation for 30 min at 1400 g prior to the analytical work.

Poly- α 1CB6 did not separate from the meniscus and was shown to be polydisperse on gel filtration with a wide molecular weight range. Therefore the material was fractionated into high- and low-molecular-weight components by gel filtration on Sepharose 4B in buffer 1. A sample (100 mg) was fractionated on a column with dimensions 87 cm $\times$ 1.6 cm. Several fractions were collected and were investigated by ultracentrifugation in buffer 1 under the same conditions as described above.

Estimation of Stokes' radius (R_s)

Poly- α 1CB6 was chromatographed on Sepharose 4B (62 cm $\times$ 1 cm) in both buffer 1 and buffer 2. The material ran as a very broad asymmetrical peak in both cases and maximum and minimum elution volumes were measured. The void (V_0) and included ($V_{inc.}$) volumes of the column were estimated using Blue Dextran and [14 C]leucine (The Radiochemical Centre, Amersham, Bucks., U.K.) respectively and the mean pore radius (r) for Sepharose 4B

was calculated from the K_d (distribution coefficient) of bovine fibrinogen (Sigma) from the following equations (Ackers, 1964):

$$K_d = \frac{V_e^{fb.} - V_0}{V_1} \quad (1)$$

where $V_e^{fb.}$ = elution volume of bovine fibrinogen, V_0 = observed void volume and $V_1 = V_{inc.} - V_0$.

$$K_d = [1 - (R_s/r)]^2 [1 - 2.104(R_s/r) + 2.09(R_s/r)^3 - 0.95(R_s/r)^5] \quad (2)$$

where R_s in this case is the Stokes' radius of fibrinogen. By this method r , the mean pore radius for Sepharose 4B was estimated as 48.64 nm. $K_d^{max.}$ and $K_d^{min.}$ for the poly- α 1CB6 species were calculated and maximal and minimal Stokes' radii were determined by means of eqn. (2).

Calculation of molecular weight from sedimentation velocity and Stokes' radius

Molecular weights were calculated from the observed sedimentation coefficients (s) and Stokes radii (R_s) from the classical equation:

$$M_r = \frac{6\pi\eta R_s s N_A}{(1 - \bar{v}\rho)} \quad (3)$$

where M_r = molecular weight, η = viscosity of buffer solution at 22°C, N_A = Avogadro's number, $\bar{v}$ = partial specific volume of poly- α 1CB6 and ρ = density of the buffer solution. The partial specific volume of poly- α 1CB6 was calculated to be 0.705 cm³ from amino-acid-analysis data (see the Results section) by the method described by Cohn & Edsall (1943).

Amino acid analysis

Samples to be analysed for amino acid content were hydrolysed *in vacuo* at 108°C for 24 h in 6 M-HCl. After drying *in vacuo*, the samples were analysed on a Locarte or a Jeol amino acid analyser. High and low molecular weight fractions of poly- α 1CB6 were prepared for amino acid analysis by gel filtration on Sepharose 4B in buffer 1 as described above.

Carbohydrate analysis

Carbohydrate analysis of poly- α 1CB6 was carried out by means of the g.l.c. method described by Clamp (1974). Interference by minor lipid contaminants was eliminated by a preliminary extraction of the sample.

The presence of galactose in α 1CB6 was investigated as follows. Mature bovine tendon (10 g) was digested with pepsin (Worthington Chemical Co.,

NJ, U.S.A.) at a ratio of 1:10 (enzyme/substrate, w/w) in 0.5 M-acetic acid for 48 h at 20°C. The soluble collagen was separated by centrifugation from residual insoluble material and was freeze-dried. The pepsin-solubilized material (1g) was digested with CNBr as described, and dried. The dry material was taken up in 0.1 M-sodium phosphate buffer, pH 5.2, and treated with 50 mg of KBH_4 for 1 h at room temperature. The solution was dialysed against 0.02 M-sodium phosphate, pH 7.4, containing 0.15 M-NaCl and then was labelled with 1 mg of galactose oxidase (Sigma) and 10 mCi of $[^3\text{H}]\text{KBH}_4$ (The Radiochemical Centre, Amersham, Bucks., U.K.) for 2 h at 20°C. After dialysing exhaustively against distilled water the material was freeze-dried and a portion was taken up in SDS solution (2%, w/v) for gel electrophoresis analysis. Gels were stained for protein and scanned prior to location of labelled peptides. This was achieved by cutting each gel into 1 mm sections, dissolving each in H_2O_2 (30% w/v) and scintillation counting in Insta-gel scintillant (Packard Instrument Co., Downers Grove, IL, U.S.A.).

Labelled $\alpha 1\text{CB6}$ was prepared as pure peptide from the remainder of the labelled CNBr peptide mixture by the methods described by Rauterberg & Kühn (1971) and 20 mg was hydrolysed in 2 M-NaOH for 48 h at 108°C. Labelled residues were identified by ion-exchange chromatography against standards in the pyridine/formic acid system described by Bailey *et al.* (1970).

Analysis of lipid content of pure and partially purified poly- $\alpha 1\text{CB6}$

CNBr digests of unwashed and acid/urea-washed (Light & Bailey, 1979b) bovine tendon were subjected to gel filtration on A-1.5m agarose in buffer 1 and the void volume (containing poly- $\alpha 1\text{CB6}$) was pooled, exhaustively dialysed against distilled water and freeze-dried. Poly- $\alpha 1\text{CB6}$ was also prepared in this way from CNBr digests of unwashed tendon, which were delipidated as described below prior to gel filtration. A sample of each of these crude poly- $\alpha 1\text{CB6}$ preparations was taken up in 5% (w/v) SDS containing 0.125 M-Tris/HCl (pH 6.8)/10% (v/v) glycerol and later analysed by SDS/polyacrylamide-gel electrophoresis. The bulk of each sample was then extracted sequentially with 20 vol. of ethanol/chloroform (3:1, v/v), ethanol/diethyl ether (3:1, v/v) and diethyl ether. The protein residue was dried under vacuum and the extracted lipid was concentrated by rotary evaporation at 25°C and was stored in ether at -22°C. Fatty acid content in saponifiable fat was analysed by g.l.c. Lipid-extracted poly- $\alpha 1\text{CB6}$ from each of the samples was also analysed by SDS/polyacrylamide gel electrophoresis as described above.

Results

Anomalous behaviour of poly- $\alpha 1\text{CB6}$ on gel filtration

The anomaly in the observed behaviour of poly- $\alpha 1\text{CB6}$ on gel filtration is exemplified by the data in Fig. 1. When CNBr-digested mature tendon was chromatographed on A-1.5m agarose (exclusion limit 1.5×10^6) in buffer 1, poly- $\alpha 1\text{CB6}$ migrated at the void volume (Fig. 1a). When a sample of this void volume material, untreated in any way and still in the running buffer, was immediately re-chromatographed on the same column of A-1.5m agarose equilibrated in the same buffer it migrated again at the void volume. However, when a portion of the same sample, collected at the void volume of the first A-1.5m column, was re-chromatographed without pre-treatment on Sepharose 4B (exclusion limit 5×10^6) in the same buffer it chromatographed over a wide range of elution volumes concomitant with a wide molecular weight range (Fig. 1b).

The yield of poly- $\alpha 1\text{CB6}$ (determined from dry wt.) at the void volume of the A-1.5m column was lower at pH 3.0 ($6.5 \pm 1.2\%$) than at pH 7.5 ($10.8 \pm 0.9\%$) and considerably lower at pH 9.5 ($4.2 \pm 0.7\%$). (Yields were calculated from the data from two experiments.) The mean pore radius of the agarose was calculated from the observed elution behaviour of bovine serum albumin as 3.0, 2.9 and 3.1 nm at pH 3.0, 7.5 and 9.5 respectively.

Fig. 2a shows that the yield of poly- $\alpha 1\text{CB6}$ (determined from dry wt.) at the void volume was dramatically decreased when the CNBr digest was chromatographed in 4 M-urea (32% of yield in 1 M- CaCl_2) whereas when the chromatography was carried out in 1 M-NaCl (Fig. 2b) $8.5 \pm 0.9\%$ of the total material migrated at the void volume (65% of yield in 1 M- CaCl_2). SDS/polyacrylamide-gel electrophoresis of the fractions collected after both experiments confirmed that in 1 M-NaCl poly- $\alpha 1\text{CB6}$ was largely collected at the void volume, whereas in 4 M-urea it was spread over a wider range of elution volumes (not shown). An increasing amount of poly- $\alpha 1\text{CB6}$ was collected at the void volume of A-1.5m agarose, with increasing protein concentration, to a maximum at 50 mg/ml (Table 1). Although the amount of CNBr-digested material remaining undissolved also increased with increasing concentration, the levels were acceptable with only 3.8% of the total solid undissolved in the 50 mg/ml solution.

Modified procedure for the preparation of pure poly- $\alpha 1\text{CB6}$

In the modified purification method presented here we could process 10 g of CNBr-digested type-I tendon collagen in each gel-filtration run (Fig. 3a)

Fig. 1. Anomalous behaviour of poly- α 1CB6 on gel filtration

(a) Gel filtration of CNBr-digested 10-year-old bovine tendon on a A-1.5m agarose column (40 cm $\times$ 1.6 cm) in buffer 1. V_0 , void volume; the bar indicates pooled material. (b) Gel filtration of half of pooled material from (a) on a Sepharose 4B column (60 cm $\times$ 1 cm) in buffer 1. In each case, ---- represents the elution profile of type-I collagen α -chains in the same buffer.

and, because of the high flow rates obtained, two runs could be carried out in one day. The yield from this step was high ($88 \pm 2.2\%$). The poly- α 1CB6 prepared by this procedure contained some other CNBr peptides, notably α 2CB3.5 (see Fig. 4). This was then removed, after passage through DEAE-Sepharose CL-6B to remove acidic protein contaminants (total losses 3–7%), by a second gel filtration separation on Sepharose 4B (Fig. 3b). In this case most of the poly- α 1CB6 migrated in a manner concomitant with its known molecular weight range (see below), chromatographing over a much wider range of elution volumes than would be

expected from its behaviour on A-1.5m agarose. A small proportion (approx. 3%) of the poly- α 1CB6 remained aggregated and eluted at the void volume of the Sepharose 4B column (Fig. 3b). The small amount of contaminating α 2CB3.5 migrated near the included volume of the Sepharose 4B and could be pooled and discarded. The remaining material accounted for up to 86% of that applied to the column. This material was defined as pure poly- α 1CB6 as it contained no detectable CNBr peptide impurities when overloaded on SDS/polyacrylamide gels (Fig. 4b), it contained no non-collagenous components by amino-acid and carbo-

Fig. 2. Influence of hydrophobic interactions on the behaviour of poly- α 1CB6 on gel filtration on A-1.5 m agarose (a) Gel filtration of a CNBr digest of 10-year-old bovine tendon on a column of A-1.5m agarose (40 cm $\times$ 1.6 cm) in 2 M-urea/0.15 M-NaCl/0.02 M-sodium phosphate, pH 7.5. (b) Gel filtration of a CNBr digest of 10-year-old bovine tendon on A-1.5m agarose (40 cm $\times$ 1.6 cm) in 1 M-NaCl containing 0.05 M-Tris/HCl, pH 7.5. Bars in (a) and (b) represent pooled fractions.

hydrate analysis (Tables 4 and 5) and its amino-acid composition was the same over its molecular weight range (Table 5).

Molecular weight estimation by SDS/polyacrylamide gel electrophoresis

Fig. 4a,b and d show the behaviour of poly- α 1CB6 on 10% (w/v), 5% (w/v) and 3–10% (w/v) gradient SDS/polyacrylamide gels. Figs. 4c and 4e show the behaviour of collagen γ , β , and α components and CNBr peptides on 5% (w/v) and 3–10% (w/v) gradient SDS/polyacrylamide gels respectively. Calibration curves were drawn for the data obtained wherein $\log$ (molecular weight) was plotted against migration (R_F) values. R_F was calculated with respect to dye-front-migration distances. Poly- α 1CB6 was shown to be polydisperse by this method with material migrating in the mol.wt. range 50 000–310 000. Diffuse peaks with mol.wts. of 72 000 and 47 500 were observed.

Estimation of molecular weight by gel filtration

Gel filtration of poly- α 1CB6 in buffer 1 on Sepharose 4B showed the material to be heterogeneous with respect to molecular weight (Fig. 5). Collagen γ , β and α chains as well as the α_2 chain

CNBr peptide α 2CB3,5 all migrated with apparently much higher molecular weights than expected (with respect to the migration behaviour of fibrinogen) as previously described (Scott & Veis, 1976). However, these collagen components did migrate in a linear manner with respect to their known molecular weights and a standard curve of $\log_{10}$ (molecular weight) versus elution volume was drawn from these data (obtained from two experiments). Using this calibration curve it was shown that the range of molecular weight spanned by the poly- α 1CB6 species was 40 000–350 000.

By scaling up this procedure as described in the methodology for the bulk preparation of poly- α 1CB6 it was possible to collect fractions of different molecular weight, dialyse and freeze-dry them and estimate the percentage by weight of the polymeric material in each fraction. The results are shown in Table 2. As can be seen more than 70% of the material lies in the mol.wt. range 115 000–350 000. Only 3.1% of the material had a mol.wt. of greater than 350 000 and 6.7% of less than 78 000.

Estimation of molecular weight by measurement of sedimentation coefficients and Stokes' radii

Poly- α 1CB6 was analysed by velocity sedimentation at 49 000 rev./min and 22°C in both

Fig. 3. Modified preparation of poly-α1CB6

See the Materials and Methods section for experimental details. (a) Gel filtration of 10g of CNBr-digested mature bovine tendon on a A-1.5m agarose column (14.5 cm × 1.6 cm) in buffer 1. The bar indicates pooled material; V_0 , void volume. (b) Gel filtration of partially purified poly-α1CB6 (V_0 peak from Fig. 3a after DEAE-Sepharose CL6B chromatography) on Sepharose 4B (55 cm × 5 cm) in buffer 1. Bars represent pooled fractions 1–6; figures in parentheses show typical percentage yields of each fraction; ----, elution profile of pure α2CB3,5 in buffer.

Table 1. Aggregation of poly-α1CB6 as a function of concentration

The results show the means obtained from two experiments. Samples 1–4 contained 100, 50, 25 and 10mg of CNBr digest/ml respectively. A-1.5m-included material accounts for all material chromatographed except for that collected in the void volume.

Fraction	Percentage of total sample recovered			
	1	2	3	4
40000 × g Pellet	6.3	3.8	3.0	1.8
A-1.5 m void-volume material	9.8	10.6	7.1	5.4
A-1.5 m included material	83.9	85.6	89.9	92.8

buffer 1 and buffer 2. Sedimentation coefficients of 3.58×10^{-13} (in buffer 1) and 2.35×10^{-13} (in buffer 2) were obtained. The discrepancy in the figures is, perhaps, an indication of the difficulty in obtaining a realistic figure for such a heterogeneous peptide mixture. In fact, in both cases, a good deal of the material did not separate from the meniscus, indicating its polydisperse nature.

Fig. 6 shows the behaviour of poly-α1CB6 and fibrinogen on gel filtration on Sepharose 4B in buffer 1. Similar profiles were obtained when chromatography was carried out in buffer 2. The void volume and included volume measurements were made with Blue Dextran and [^{14}C]leucine respectively. The

Fig. 4. SDS/polyacrylamide gel electrophoresis of highly purified poly- α 1CB6

Scans of Coomassie Brilliant Blue stained gels are shown. (a) Poly- α 1CB6 electrophoresed in a gel containing 5% (w/v) acrylamide; (b) poly- α 1CB6 electrophoresed in a gel containing 10% (w/v) acrylamide; (c) bovine skin type-I collagen chains electrophoresed in a gel containing 5% (w/v) acrylamide; (d) poly- α 1CB6 electrophoresed in a gel consisting of a gradient of 3–10% (w/v) acrylamide; (e) bovine skin type-I collagen chains and CNBr peptides electrophoresed in a gel consisting of a gradient of 3–10% (w/v) acrylamide.

Stokes radius (R_s) for fibrinogen was taken as 11.0nm (Siegel & Monty, 1966). By application of eqn. (1), K_d for fibrinogen was calculated as 0.388 in buffer 1 and 0.333 in buffer 2. From the values of K_d and R_s and application of eqn. (2) the mean pore size of the agarose in buffer 1 was calculated as 54.3nm and in buffer 2 48.6nm. Utilizing these figures and the observed upper and lower limits of K_d for the poly- α 1CB6 in both buffers, values for the maximum and minimum Stokes' radii for the material were calculated. These were then applied to eqn. (3) to calculate the molecular weight ranges. These were found to be 46 000–270 000 in buffer 1 and 50 000–140 000 in buffer 2. These data are summarized in Table 3.

Owing to the discrepancy in calculated molecular

weight, the velocity sedimentation coefficients of fractions of high and low molecular weights collected from a Sepharose 4B column (see above) were determined in buffer 1 by analytical ultracentrifugation as described. The average Stokes' radius for each fraction was also calculated from the observed elution volumes and column parameters as before and from these values the molecular weight of polymers in each fraction was calculated. The results are shown in Table 3. As can be seen, the results correlate well with the upper and lower limits of molecular weight calculated by the methods described above. In each case the peak obtained on velocity sedimentation was much clearer than that obtained with whole poly- α 1CB6 and thus a more accurate measurement of velocity sedimentation coefficient was possible.

Amino-acid analysis

Table 4 shows a comparison of the amino acid compositions of various theoretical cross-linked peptide combinations with that of highly purified poly- α 1CB6. As can be seen, cross-linked peptides containing higher proportions of α 1CB5 than α 1CB1 show a much better fit with the observed poly- α 1CB6 amino-acid composition. In fact, the closest parallel to the poly- α 1CB6 analysis was obtained with the α 1CB6– α 1CB5 cross-linked peptide. It should be noted that, as approximately 50% of α 1CB6 has lost several residues from the C-terminus (Rauterberg & Kühn, 1971; Light & Bailey, 1979b), the amino-acid composition of α 1CB6 used for calculation of theoretical cross-linked peptide combinations was taken to be the average of a 1:1 mixture of whole α 1CB6 (α 1CB6a) and the so-called α 1CB6b.

Molecular weight fractionation of pure poly- α 1CB6 by gel filtration on Sepharose 4B gave five fractions which were pooled, dialysed and freeze-dried prior to determination of their amino-acid content. The results of this experiment are detailed in Table 5. No significant differences in amino acid content were observed between the various fractions. No fraction contained significant amounts of methionine, indicating that the polydisperse nature of poly- α 1CB6 was not due to the presence of CNBr-uncleaved peptide.

Carbohydrate analysis

Carbohydrate analysis of purified, delipidated poly- α 1CB6 showed that the material contained only residues of galactose and glucose, as expected for purified type-I-collagen derived material. When the carbohydrate content was calculated as a percentage of the total material, figures of 0.72 and 0.28% (w/w) for galactose and glucose respectively were obtained. As poly- α 1CB6 is a polydisperse species it was impossible to determine the number of

Table 2. *Distribution of different sized molecular weight species of poly- α 1CB6*

A heterogeneous preparation of poly- α 1CB6 was separated with respect to molecular size by gel filtration in buffer 1 on Sepharose 4B (see text for details). Five fractions [not including fraction 1, the void volume material (accounting for approximately 3% of total material)] were pooled, dialysed, freeze-dried and weighed. The molecular weight ranges of the species in each fraction were determined using collagen standards chromatographed on the same column (see Fig. 3b). The last column contains results averaged from two experiments.

Fraction no.	$10^{-3} \times$ Mol.wt. range	Percentage (by wt.)
2	350–450	3.1
3	180–350	37.0
4	115–180	35.1
5	78–115	18.2
6	44–78	6.7

galactose and glucose residues per poly- α 1CB6 molecule.

In each case, galactose peaks were found to be superimposed on a broad, trailing unknown peak. This made accurate calculation of galactose levels impossible and thus, all galactose values are the mean of upper and lower limits calculated from the trace. The broad peak and another eluting coincident with one of the *N*-acetylglucosamine peaks were unknown components, accounting for at least an equal amount of material as represented by the carbohydrate. These peaks were not due to lipid as the poly- α 1CB6 was extracted with organic solvents before and after volatilization. These components require further investigation.

Pure poly- α 1CB6 was fractionated with respect to molecular weight by gel filtration and the various fractions obtained were analysed for carbohydrate content. The results are shown in Table 5. As can be

Table 3. *Measurement of sedimentation velocity and Stokes' radii for poly- α 1CB6 species*
Buffer 1 is 1 M-CaCl₂/0.05 M-Tris/HCl, pH 7.5. Buffer 2 is 2 M-urea/0.15 M-NaCl/0.02 M-sodium phosphate, pH 7.5.

Protein	Observed <i>s</i> (10^{-13} s) in		Observed <i>K_d</i> in		Observed <i>R_s</i> (nm) in		$10^{-3} \times$ Calculated mol. wt. in	
	Buffer 1	Buffer 2	Buffer 1	Buffer 2	Buffer 1	Buffer 2	Buffer 1	Buffer 2
Fibrinogen	—	—	0.388	0.333	11.0*	11.0*	—	—
Poly- α 1CB6	3.58	2.35	0.3–0.85	0.27–0.66	2.2–13	4.6–12.6	46–270	50–140
High-molecular-weight poly- α 1CB6†	4.29	—	0.29	—	12.8	—	320	—
Low-molecular-weight poly- α 1CB6†	3.00	—	0.84	—	2.6	—	45	—

* From Siegel & Monty (1966).

† Separated by gel filtration in buffer 1 (see text).

Fig. 5. *Determination of maximum and minimum molecular weights of poly- α 1CB6 by gel filtration*
Gel filtration on Sepharose 4B (60 cm $\times$ 1 cm) in buffer 1. —, Elution profile for type-I collagen chains and α 2CB3,5 in buffer 1. See the Materials and Methods section for experimental details.

Fig. 6. Determination of maximum and minimum Stokes' radii (R_s) for poly- α 1CB6 in buffer 1. Gel filtration of pure poly- α 1CB6 on Sepharose 4B (60 cm $\times$ 1 cm) in buffer 1 is shown. ----, Elution profile of pure bovine fibrinogen in buffer 1.

Table 4. A comparison of the observed amino acid composition of pure poly- α 1CB6 with the compositions of several possible theoretical cross-linked peptides

1, α 1CB6- α 1CB5; 2, $(\alpha$ 1CB6) $_3$ -(α 1CB5) $_2$; 3, $(\alpha$ 1CB6) $_2$ - α 1CB5; 4, $(\alpha$ 1CB6) $_3$ - α 1CB5- α 1CB1; 5, $(\alpha$ 1CB6) $_2$ - α 1CB5- α 1CB1; 6, $(\alpha$ 1CB6) $_3$ - α 1CB5-(α 1CB1) $_2$; 7, α 1CB6- α 1CB5- α 1CB1; 8, α 1CB6- α 1CB1. All amino acid compositions were calculated using the amino acid sequence of the peptides concerned (Piez, 1976). Hse, homoserine. N.D., not determined.

Amino acid	Composition (residues/1000 residues) of								
	Poly- α 1CB6*	1	2	3	4	5	6	7	8
Hyp	87.5	90.9	91.4	91.7	89.7	88.5	87.6	85.2	85.8
Asp	51.4	51.4	49.9	49.0	48.4	49.4	48.7	51.9	47.2
Thr	16.0	11.9	12.5	12.8	14.2	14.4	15.3	14.8	17.2
Ser	34.1†	43.5	42.9	42.6	45.6	47.3	48.7	51.9	51.5
Hse	N.D.	4.0	2.8	2.1	2.8	4.1	4.2	7.4	4.3
Glu	72.6	75.1	74.8	74.6	74.1	74.1	73.7	74.1	73.0
Pro	134.4	134.4	138.5	140.7	142.5	139.9	141.9	133.3	145.9
Gly	336.9	316.2	315.8	315.6	312.0	310.7	308.8	307.4	304.7
Ala	105.0	102.8	102.5	102.3	99.7	98.8	97.4	96.3	94.4
Cys	—	—	—	—	—	—	—	—	—
Val	23.0	7.9	8.3	8.5	10.0	10.3	11.1	11.1	12.9
Met	—	—	—	—	—	—	—	—	—
Ile	14.0	11.9	12.5	12.8	14.2	14.4	15.3	14.8	17.2
Leu	26.0	27.7	27.7	27.7	28.5	28.8	29.2	29.6	30.0
Tyr	3.6†	11.9	12.5	12.8	15.7	16.5	18.1	18.5	21.5
Phe	13.2	11.9	11.1	10.7	10.0	10.3	9.7	11.1	8.6
Hyl	8.7	7.9	8.3	8.5	8.5	8.2	8.3	7.4	8.6
Lys	21.9	23.7	22.2	21.3	19.9	20.6	19.5	22.2	17.2
His	5.8	11.9	11.1	10.7	10.0	10.3	9.7	11.1	8.6
Arg	47.7	51.4	52.6	53.3	52.7	51.4	51.5	48.1	51.5

* Averaged from ten determinations.

† Not corrected for losses during hydrolysis.

seen, the only carbohydrates present in any fraction were galactose and glucose, as initially found in whole poly- α 1CB6. The relative amounts of galactose per unit of glucose increased slightly with

increasing molecular weight and both monosaccharides were found in every fraction.

Fig. 7 shows gel scans and radioactivity profiles for galactose oxidase/ KB^3H_4 -labelled CNBr pep-

Table 5. Amino acid and carbohydrate composition of poly- α 1CB6 fractions separated by gel filtration on a basis of molecular weight

For experimental details of fractionation and molecular weight range of each fraction see the Materials and Methods section and Table 2. The galactose/glucose ratio was calculated from g.l.c. analysis data (see the text). N.D., not determined. Hse, homoserine.

Amino acid	Composition (residues/1000 residues) of fraction				
	2	3	4	5	6
Hyp	79.1	80.8	81.8	78.2	80.6
Asp	51.2	52.6	51.4	49.2	50.0
Thr	16.6	16.5	16.1	16.5	15.8
Ser	40.4	38.9	40.1	39.1	38.0
Hse	N.D.	N.D.	N.D.	N.D.	N.D.
Glu	70.7	65.8	70.8	69.2	71.0
Pro	126.2	153.8	140.2	131.8	138.7
Gly	337.0	319.5	337.4	339.8	344.0
Ala	108.8	100.0	98.1	104.5	107.0
Cys	—	—	—	—	—
Val	24.6	21.1	22.1	21.8	22.2
Met	—	—	—	—	—
Ile	14.8	14.9	14.6	15.9	12.8
Leu	28.5	28.1	26.1	27.5	25.0
Tyr	5.3	5.6	5.2	5.0	3.7
Phe	13.3	14.6	12.0	13.0	12.1
Hyl	8.3	7.1	7.7	8.4	8.2
Lys	20.7	20.4	19.4	21.7	20.8
His	5.6	7.6	7.0	7.0	5.4
Arg	48.9	50.8	50.0	51.4	46.1
Galactose/glucose ratio	3.5:1	5:1	4.2:1	2.9:1	3.4:1

Fig. 7. SDS/polyacrylamide-gel electrophoresis of CNBr peptides of pepsin-solubilized mature bovine tendon collagen labelled by the galactose oxidase method

See the Materials and Methods section for experimental details. Scan of Coomassie Brilliant Blue-stained gel containing 10% (w/v) acrylamide is shown. ----, Radioactivity assayed by liquid scintillation spectrometry.

Lipid content of pure and partially purified poly- α 1CB6

Poly- α 1CB6, purified from unwashed mature bovine tendon by a single A-1.5m agarose-gel filtration step, contained small amounts of saponifiable lipid (Table 6). The fatty acids present on g.l.c. analysis were myristic acid (C_{14}), palmitic acid (C_{16}), palmitoleic acid ($C_{16:1}$), stearic acid (C_{18}), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and trace amounts of C_{20} and C_{22} fatty acids. Total lipid accounted for $0.6 \pm 0.02\%$ by wt. of poly- α 1CB6 in these preparations. The lipid content of poly- α 1CB6 was significantly decreased when the tendon was extensively washed with 4M-urea in 0.5M-acetic acid prior to preparative procedures (0.11% lipid). Although very little variation in fatty acid content was seen in any of the samples examined, the level of stearate as a percentage of other fatty acids was significantly higher in poly- α 1CB6 than in the whole CNBr digest. Even higher proportions of this fatty acid were seen in poly- α 1CB6 prepared from acid/urea-washed tendon.

Discussion

Poly- α 1CB6 exhibited unusual and anomalous characteristics on gel filtration. Although eluting

tides from pepsin-treated collagen. As can be seen, α 1CB6 contains a significant proportion of the label. This was shown to be hydroxylysine-galactose by analytical techniques.

Table 6. *Lipid content of poly- α 1CB6*

Poly- α 1CB6 was partially purified by A-1.5m agarose gel filtration. The lipid content of the protein was compared when derived from urea-washed and unwashed bovine tendon and with the lipid content of CNBr-digested unwashed tendon. Sample 1, CNBr digest of unwashed mature bovine tendon; sample 2, poly- α 1CB6 from unwashed tendon; sample 3, poly- α 1CB6 from unwashed tendon (second preparation); sample 4, poly- α 1CB6 from acid/urea washed tendon. See text for details.

Sample	Fatty acid content (% of total analysis)							Total % of fat (by wt.)
	Myristate (C ₁₄)	Palmitate (C ₁₆)	Palmitoleate (C _{16:1})	Stearate (C ₁₈)	Oleate (C _{18:1})	Linoleate (C _{18:2})	Others	
1	1.6	16.8	10.0	3.8	64.4	1.2	2.2	1.77
2	2.2	21.7	7.8	6.7	56.4	0.9	4.3	0.62
3	3.5	27.1	8.9	5.8	50.2	1.0	3.5	0.58
4	1.8	19.1	7.4	9.5	58.0	1.2	3.0	0.11

over a wide range of volumes on Sepharose 4B, indicating a mol.wt. range of 50000–350000 (see below), it eluted predominantly at the void volume of A-1.5m consistent with a mol.wt. in excess of 350000 (Fig. 1). The data obtained from studying the chromatographic behaviour of poly- α 1CB6 on A-1.5m agarose under a variety of conditions suggest that it interacts with the matrix in such a way as to inhibit its entry into the pores. When chromatographed on A-1.5m in 4M-urea (Fig. 2a) it exhibited the molecular weight range and elution pattern expected from its behaviour on Sepharose 4B, although in 1M-CaCl₂ (Fig. 1a) or 1M-NaCl (Fig. 2b) it eluted predominantly at the void volume. Similarly, although the mean pore diameter of the A-1.5m agarose remained relatively unaltered by changes in pH the elution pattern of poly- α 1CB6 changed such that at extremes of pH more poly- α 1CB6 was incorporated into the matrix. Presumably the high net charge on the peptides at low and high pH decreased their tendency to interact with the agarose.

Therefore, we conclude that, due to the polymeric nature and possibly unusual shape of the poly- α 1CB6 oligomers, this cross-linked peptide material interacts across the pores of A-1.5m agarose, probably by means of hydrophobic or hydrogen bonds rather than ionic associations, in a way which limits its ability to enter the agarose matrix. This interaction is also apparently dependent on total protein concentration as yields of poly- α 1CB6 at the A-1.5m void volume were optimal at 50mg/ml and were decreased at concentrations lower than this (Table 1). This unusual phenomenon proved a useful tool in the purification of poly- α 1CB6.

We obtained yields of up to 70% of the expected theoretical quantities of poly- α 1CB6 using the modified method reported here and the poly- α 1CB6 prepared by this procedure contained no other known peptide contaminants by the criteria of SDS/polyacrylamide-gel electrophoresis and amino-acid analysis.

It is clear from the results presented here that the C-terminal cross-linked peptide, poly- α 1CB6, contains a wide range of different sized molecules. Determination of molecular size in such a heterogeneous mixture presents obvious difficulties. However, we have utilized three independent methods for the estimation of the upper and lower molecular weight limits of poly- α 1CB6 and the correlations obtained were good. The molecular weight ranges covered by the poly- α 1CB6 species were determined as 47000–310000 by SDS/polyacrylamide-gel electrophoresis (Fig. 4), 40000–350000 by gel filtration (Fig. 5) and 46000–270000 by determination of sedimentation coefficient and Stokes' radius in buffer 1 and 50000–140000 by the same measurements made in buffer 2 (Table 3). Although the upper limit figure for the last method in buffer 2 is low compared with the other three sets of data, we can conclude with confidence that the poly- α 1CB6 molecular species span a mol.wt. range of approx. 50000–350000.

By separating the various molecular weight fractions of poly- α 1CB6 by gel filtration it was possible to quantify the amount of material in each range (Table 2). High-molecular-weight polymers (over 350000) were not common, only accounting for 3% of the total material. Material with a mol.wt. of less than 115000 accounted for 25% of the pure poly- α 1CB6. Polymers in the mol.wt. range 115000–350000, however, accounted for 72% of the material. The amino acid compositions of all polymers of poly- α 1CB6 were the same (within the limits of experimental error) showing the material to be chemically homogeneous (Table 5).

Poly- α 1CB6 contained only collagenous protein material from its amino-acid composition (Table 4) and no significant proportions of any carbohydrates except small amounts of galactose and glucose. By these criteria the peptide material was not contaminated by, or associated with, any non-collagenous proteins or glycoproteins. Therefore, the observed unusual gel pattern cannot be due to the

presence of large amounts of carbohydrate or carbohydrate-containing proteins, which are known to sometimes electrophorese as very broad, fuzzy bands. Similarly, the lipid content of poly- α 1CB6 is low (less than 1% in samples derived from unwashed tendon and only 0.11% in samples derived from washed tendon); thus the possibility of lipid contaminants causing the observed gel pattern is unlikely. Indeed, delipidated poly- α 1CB6 gave exactly the same result on SDS/polyacrylamide-gel electrophoresis as non-delipidated samples (not shown).

Our data suggest (see below) that poly- α 1CB6 contains a mixture of α 1CB6 and α 1CB5, probably in a ratio of 2:1 or 3:1. Thus the observed micro-heterogeneity of poly- α 1CB6 on SDS/polyacrylamide gels may be explained by the number of possible associations of each α 1CB6 dimer, trimer, tetramer, etc. with α 1CB5. Even greater diversity in the type and size of α 1CB6 oligomers may be expected, due to the known propensity of the peptide to lose amino acids from the C-terminal end during preparation procedures (Rauterberg & Kühn, 1971). Monomeric α 1CB6 has been shown to migrate on SDS/polyacrylamide gels as an ill-defined doublet (Light & Bailey, 1979b) or even triplet (Glanville *et al.*, 1979).

Poly- α 1CB6 contained small amounts of lipid that could be removed by washing the pure protein with organic solvents (Table 6). The fatty acid content of this lipid was similar in type and proportions to the usual animal-fat fatty acid components. However, differences were observed when poly- α 1CB6 was prepared from washed and unwashed tendon. A 3-fold increase in stearate content was seen when the fatty acids present in pure poly- α 1CB6 prepared from washed tendon was compared with those present in the crude CNBr digest of unwashed tendon. No changes in any of the other fatty acid levels were observed. This may indicate a specific association of poly- α 1CB6 (or the C-terminal region of type-I collagen) with stearic-acid-rich lipids. However, the results are only indicative and this suggestion needs further confirmation.

Previous work has shown that only α 1CB6, α 1CB5 and α 1CB1 are likely to be involved in cross-linking type-I collagen (Kang, 1972; Henkel *et al.*, 1976; Light & Bailey, 1979b; Light, 1979). Table 4 compares the calculated amino acid compositions of various combinations of these peptides that may be expected to lead to the formation of poly- α 1CB6 with the mean values for amino acid composition obtained from analysis of the highly purified cross-linked polymeric material. It should be noted that this latter analysis differs slightly from that previously published (Light & Bailey, 1980) due to the higher purity of the present preparation.

The observed value for valine was found to be approximately 100% higher than the calculated values. At the present time this abnormal valine content cannot be accounted for. The material did not contain measurable amounts of methionine, indicating that its polydisperse nature was not due to incomplete CNBr cleavage.

Comparing poly- α 1CB6 with a 1:1 co-polymer of α 1CB6 and α 1CB5 (Henkel *et al.*, 1976) the calculated amino acid composition showed a good fit with the observed values. Only threonine, isoleucine, tyrosine and histidine showed variations from the observed values. However, considering poly- α 1CB6 to be a 1:1 co-polymer of α 1CB6 and α 1CB1 (Kang, 1972), very large variations from the observed amino acid composition were seen, with only eight values falling within 10% of those observed, and isoleucine, tyrosine, phenylalanine and lysine showing large deviations (Table 4).

Of the various more complex cross-linked peptide combinations possible ($(\alpha$ 1CB6)₃-(α 1CB5)₂ and (α 1CB6)₂-(α 1CB5)₁, showed the best fit with the amino acid composition of poly- α 1CB6. As increasing amounts of α 1CB1 were added to the theoretical peptide combinations less and less comparability was seen between the observed and calculated levels. However, the 1:1:1 co-polymer of α 1CB6, α 1CB5 and α 1CB1, although showing gross differences in tyrosine and histidine as well as increases in leucine, phenylalanine and hydroxylysine, gave the third best fit with the observed amino-acid composition of pure poly- α 1CB6.

Under these rigorous comparative conditions the (α 1CB6)₃-(α 1CB5)₂ and (α 1CB6)₂-(α 1CB5)₁ copolymers showed an impressive similarity to the amino-acid content of poly- α 1CB6, further supporting the proposed peptide origin of the polymeric cross-linked species.

The smallest possible unit of the polymeric cross-linked C-terminal material that can be postulated to fit the amino-acid-analysis data is (α 1CB6)₂-(α 1CB5)₁, which has a mol.wt. of approx. 43 400. This figure is in good agreement with the lowest molecular weight material observed in the poly- α 1CB6 preparations.

Whole poly- α 1CB6 contained galactose and glucose in a molar ratio of 2.6:1. When separated into different molecular weight fractions the lower-molecular-weight range oligomers contained these two monosaccharides in a molar ratio of 3.4:1 and 2.9:1 (for fractions of mol.wt. range 44 000–78 000 and 78 000–115 000 respectively). We know that α 1CB5 contains one residue each of galactose and glucose (Butler, 1969) and that α 1CB6 contains one residue of galactose from the analytical work described in this report. Therefore, the carbohydrate analysis data is in good agreement with the above suggestion of (α 1CB6)₂-(α 1CB5)₁ being a basic

structural unit, as this peptide would contain galactose and glucose in the ratio 3:1.

The higher-molecular-weight fractions of poly- α 1CB6 showed a galactose/glucose ratio of 4:1 and 5:1, indicating higher proportions of α 1CB6 in these oligomers. It should be remembered, however, that accurate measurements of galactose were impossible to obtain due to the extra, unknown component which co-chromatographed with this monosaccharide on g.l.c., so that the interpretation of these data should be viewed with some caution.

We suggest that the quarter-staggered penta-fibrils, cross-linked internally in immature tissue by reducible covalent aldimine and ketoimine bonds, are aligned in register within the fibril. In this way a reactive residue(s) in the C-terminal (α 1CB6) of a molecule in one penta-fibril would align with a residue, probably a reducible cross-link, in the adjacent penta-fibril and may react to form a trivalent (multivalent) stable cross-link. The final stable cross-link may in fact be multivalent and involve several α chains, but the initial requirement of a second α 1CB6 in a molecule in an adjacent penta-fibril coming into reaction distance with a primary α 1CB6 containing a reducible cross-link in a separate penta-fibril has to be fulfilled in order to explain the postulated (α 1CB6)₂-(α 1CB5)₁ monomer. This reaction must produce a non-reducible stable bond in order to account for the accumulation of poly- α 1CB6 in mature tissue, the decrease of reducible cross-links on maturation and the concomitant increase in stability of the collagen.

Evidence for such a precise in-register alignment of penta-fibrils is available from recent X-ray work (Fraser *et al.*, 1979). The alignment of quarter-staggered penta-fibrils in register is obviously necessary in order to lead to the well-described banded pattern observed in electron micrographs of collagen fibrils. Quarter-staggered penta-fibrils packed within the fibril out of exact alignment with each other would not lead to this regularly banded pattern.

The data in this report demonstrates that 72% of the poly- α 1CB6 prepared has a mol.wt. of between 115 000–350 000 (Table 2). From five to seventeen α 1CB6 peptides must be involved in forming such oligomers, i.e. three to eight (α 1CB6)₂-(α 1CB5)₁ units. When each stable bond, linking two penta-fibrils, is formed, a single α 1CB5 must also become covalently associated with the α 1CB6 peptides involved, due to its participation in the formation of reducible cross-links as explained above. By means of specific orientation of the penta-fibrils, brought about by the interaction of side chains on their outer edges, aligned molecules could form stable cross-links that might be expected to link a number of molecules in different penta-fibrils. Chain reactions of this sort may then lead to the formation of higher

molecular weight aggregates containing 15–17 α 1CB6 peptides, with the upper limit probably determined by steric and positional limitations. A theoretical model based on the available data to test these suggestions would be of interest.

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