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-α1CB6) 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-α1CB6 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 α1CB6 and α1CB5. The material was shown to be polydisperse with a mol.wt. range of 50000–350000, 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-α1CB6 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-α1CB6 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 (α1CB6)2–(α1CB5), may be the basic cross-linked structural unit of poly-α1CB6. Oligomers of poly-α1CB6 therefore may contain from one to eight (α1CB6)2–(α1CB5), units, although the carbohydrate analysis indicated that the higher molecular weight oligomers may be enriched in α1CB6.

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 α1CB6, the α1 chain C-terminal peptide. This material, termed poly-α1CB6, consisted predominantly of α1CB6 and was stable to treatment by periodate.

In the present paper we report a modification to the original preparation procedure of poly-α1CB6 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-α1CB6 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-α1CB6 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 30 min. The solution was clarified by centrifugation at 30 min for 40000 g and 1ml was chromatographed on a column (40cm x 1.6cm) of A-1.5m agarose in buffer 1. The void volume peak (poly-α1CB6) 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 x 1.0cm) of Sepharose 4B equilibrated in buffer 1.

The behaviour of poly-α1CB6 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-α1CB6 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-α1CB6 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-α1CB6 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-α1CB6 was estimated.

Bulk preparation and purification of poly-α1CB6

In general, for bulk preparations, 10g of CNBr-digested tendon was dissolved in buffer 1 to a final concentration of 50mg/ml by incubating at 60°C for 30 min. The solution was clarified by centrifugation at 40000 g for 30 min 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-α1CB6) was pooled, dialysed exhaustively against distilled water and freeze-dried.

Partially purified poly-α1CB6 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-Sephrose CL-6B (Light & Bailey, 1980). The material was again dialysed and freeze-dried before dissolving it in buffer 1 at 60°C for 10 min. Some of the material (1g) at a concentration of 50mg/ml was loaded onto a column (55cm x 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-α1CB6

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-α1CB6 species.

(a) SDS/polyacrylamide-gel electrophoresis.

Poly-α1CB6 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 30 min 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-α1CB6 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 \( R_p \) values (with respect to dye front) against the log 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-α1CB6 (5 mg/ml in buffer 1) was chromatographed on a column (62 cm × 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-α1CB6 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, CB3,5, the α, chain C-terminal CNBr peptide.

(c) Sedimentation velocity in the analytical ultracentrifuge. The sedimentation velocity of the poly-α1CB6 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-α1CB6 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 × 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-α1CB6 was chromatographed on Sepharose 4B (62 cm × 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 [14C]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):

\[
K_d = \frac{V_{fb}^n - V_0}{V_1}
\]

where \( V_{fb} \) = elution volume of bovine fibrinogen, \( V_0 \) = observed void volume and \( V_1 = V_{inc} - V_0 \).

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

where \( R_s \) in this case is the Stokes’ radius of fibrinogen. By this method, the mean pore radius for Sepharose 4B was estimated as 48.64 nm. \( K_d^{max} \) and \( K_d^{min} \) for the poly-α1CB6 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:

\[
M_r = \frac{6\pi\eta R_s s N_A}{(1 - \delta \rho)}
\]

where \( M_r \) = molecular weight, \( \eta \) = viscosity of buffer solution at 22°C, \( N_A \) = Avogadro’s number, \( \delta \) = partial specific volume of poly-α1CB6 and \( \rho \) = density of the buffer solution. The partial specific volume of poly-α1CB6 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-α1CB6 were prepared for amino acid analysis by gel filtration on Sepharose 4B in buffer 1 as described above.

Carbohydrate analysis

Carbohydrate analysis of poly-α1CB6 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 α1CB6 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 0.5M-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 0.1M-sodium phosphate buffer, pH 5.2, and treated with 50mg of KBH4 for 1h at room temperature. The solution was dialysed against 0.02M-sodium phosphate, pH 7.4, containing 0.15M-NaCl and then was labelled with 1mg of galactose oxidase (Sigma) and 10mCi of 13H|KBH4 (The Radiochemical Centre, Amersham, Bucks., U.K.) for 2h 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 1mm sections, dissolving each in H2O2 (30% w/v) and scintillating counting in Insta-gel scintillant (Packard Instrument Co., Downers Grove, Ill., U.S.A.).

Labelled α1CB6 was prepared as pure peptide from the remainder of the labelled CNBr peptide mixture by the methods described by Rauterberg & Kühn (1971) and 20mg was hydrolysed in 2M-NaOH for 48h 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-α1CB6

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-α1CB6) was pooled, exhaustively dialysed against distilled water and freeze-dried. Poly-α1CB6 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-α1CB6 preparations was taken up in 5% (w/v) SDS containing 0.125M-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 20vol. 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-α1CB6 from each of the samples was also analysed by SDS/polyacrylamide gel electrophoresis as described above.

Results

Anomalous behaviour of poly-α1CB6 on gel filtration

The anomaly in the observed behaviour of poly-α1CB6 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 × 106) in buffer 1, poly-α1CB6 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 × 106) 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-α1CB6 (determined from dry wt.) at the void volume of the A-1.5m column was lower at pH 3.0 (6.5 ± 1.2%) than at pH 7.5 (10.8 ± 0.9%) and considerably lower at pH 9.5 (4.2 ± 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 3.1nm at pH 3.0, 7.5 and 9.5 respectively.

Fig. 2a shows that the yield of poly-α1CB6 (determined from dry wt.) at the void volume was dramatically decreased when the CNBr digest was chromatographed in 4M-urea (32% of yield in 1M-CaCl2) whereas when the chromatography was carried out in 1M-NaCl (Fig. 2b) 8.5 ± 0.9% of the total material migrated at the void volume (65% of yield in 1M-CaCl2). SDS/polyacrylamide-gel electrophoresis of the fractions collected after both experiments confirmed that in 1M-NaCl poly-α1CB6 was largely collected at the void volume, whereas in 4M-urea it was spread over a wider range of elution volumes (not shown). An increasing amount of poly-α1CB6 was collected at the void volume of A-1.5m agarose, with increasing protein concentration, to a maximum at 50mg/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 50mg/ml solution.

Modified procedure for the preparation of pure poly-α1CB6

In the modified purification method presented here we could process 10g of CNBr-digested type-I tendon collagen in each gel-filtration run (Fig. 3a)
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 ± 2.2%). The poly-α1CB6 prepared by this procedure contained some other CNBr peptides, notably α2CB3,5 (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-α1CB6 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-α1CB6 remained aggregated and eluted at the void volume of the Sepharose 4B column (Fig. 3b). The small amount of contaminating α2CB3,5 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-α1CB6 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. 1. Anomalous behaviour of poly-α1CB6 on gel filtration
(a) Gel filtration of CNBr-digested 10-year-old bovine tendon on a A-1.5m agarose column (40cm × 1.6cm) 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 (60cm × 1 cm) in buffer 1. In each case, represents the elution profile of type-I collagen α-chains in the same buffer.
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-α1CB6 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 (Rf) values. Rf was calculated with respect to dye-front-migration distances. Poly-α1CB6 was shown to be polydisperse by this method with material migrating in the mol.wt. range 50000–310000. Diffuse peaks with mol.wts. of 72000 and 47500 were observed.

**Estimation of molecular weight by gel filtration**

Gel filtration of poly-α1CB6 in buffer I 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 α2 chain CNBr peptide α2CB3,5 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 log10 (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-α1CB6 species was 40000–350000.

By scaling up this procedure as described in the methodology for the bulk preparation of poly-α1CB6 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 115000–350000. Only 3.1% of the material had a mol.wt. of greater than 350000 and 6.7% of less than 78000.

**Estimation of molecular weight by measurement of sedimentation coefficients and Stokes’ radii**

Poly-α1CB6 was analysed by velocity sedimentation at 49000rev./min and 22°C in both
POLYMERIC C-TERMINAL CROSS-LINKED MATERIAL FROM TYPE-I COLLAGEN

2.01 - 1.0

F

1000

400

2000

800

Elution volume (ml)

3000

1200

Fig. 3. Modified preparation of poly-α1CB6
See the Materials and Methods section for experimental details. (a) Gel filtration of 10 g of CNBr-digested mature bovine tendon on a A-1.5 m agarose column (14.5 cm x 1.6 cm) in buffer 1. The bar indicates pooled material; V₀, void volume. (b) Gel filtration of partially purified poly-α1CB6 (V₀ peak from Fig. 3a after DEAE-Sepharose CL6B chromatography) on Sepharose 4B (55 cm x 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 10 mg of CNBr digest/ml respectively. A-1.5 m-included material accounts for all material chromatographed except for that collected in the void volume.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>40000 x g Pellet</td>
<td>6.3</td>
<td>3.8</td>
<td>3.0</td>
<td>1.8</td>
</tr>
<tr>
<td>A-1.5 m void-volume material</td>
<td>9.8</td>
<td>10.6</td>
<td>7.1</td>
<td>5.4</td>
</tr>
<tr>
<td>A-1.5 m included material</td>
<td>83.9</td>
<td>85.6</td>
<td>89.9</td>
<td>92.8</td>
</tr>
</tbody>
</table>

buffer 1 and buffer 2. Sedimentation coefficients of $3.58 \times 10^{-13}$ (in buffer 1) and $2.35 \times 10^{-13}$ (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 [14C]leucine respectively. The
Stokes radius ($R_s$) for fibrinogen was taken as 11.0 nm (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 54.3 nm and in buffer 2 48.6 nm. Utilizing these figures and the observed upper and lower limits of $K_d$ for the poly-$\alpha$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 46000–270000 in buffer 1 and 50000–140000 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-$\alpha$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-$\alpha$1CB6. As can be seen, cross-linked peptides containing higher proportions of $\alpha$1CB5 than $\alpha$1CB1 show a much better fit with the observed poly-$\alpha$1CB6 amino-acid composition. In fact, the closest parallel to the poly-$\alpha$1CB6 analysis was obtained with the $\alpha$1CB6–$\alpha$1CB5 cross-linked peptide. It should be noted that, as approximately 50% of $\alpha$1CB6 has lost several residues from the C-terminus (Rauterberg & Kühn, 1971; Light & Bailey, 1979b), the amino-acid composition of $\alpha$1CB6 used for calculation of theoretical cross-linked peptide combinations was taken to be the average of a 1:1 mixture of whole $\alpha$1CB6 ($\alpha$1CB6a) and the so-called $\alpha$1CB6b.

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

**Carbohydrate analysis**

Carbohydrate analysis of purified, delipidated poly-$\alpha$1CB6 showed that the material contained only residues of galactose and glucose, as expected for purified type-I-collagen derived material. When the carbohydrate content was calculated as a percentage of the total material, figures of 0.72 and 0.28% (w/w) for galactose and glucose respectively were obtained. As poly-$\alpha$1CB6 is a polydisperse species it was impossible to determine the number of
Table 2. Distribution of different sized molecular weight species of poly-α1CB6
A heterogeneous preparation of poly-α1CB6 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.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>10⁻³× Mol.wt. range</th>
<th>Percentage (by wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>350-450</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>180-350</td>
<td>37.0</td>
</tr>
<tr>
<td>4</td>
<td>115-180</td>
<td>35.1</td>
</tr>
<tr>
<td>5</td>
<td>78-115</td>
<td>18.2</td>
</tr>
<tr>
<td>6</td>
<td>44-78</td>
<td>6.7</td>
</tr>
</tbody>
</table>

galactose and glucose residues per poly-α1CB6 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-α1CB6 was extracted with organic solvents before and after volatilization. These components require further investigation.

Pure poly-α1CB6 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-α1CB6 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.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Observed s (10⁻¹³ s) in Buffer 1</th>
<th>Observed s (10⁻¹³ s) in Buffer 2</th>
<th>Observed K₄ in Buffer 1</th>
<th>Observed K₄ in Buffer 2</th>
<th>Observed Rₛ in nm</th>
<th>10⁻³ × Calculated mol. wt. in Buffer 1</th>
<th>10⁻³ × Calculated mol. wt. in Buffer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>3.58</td>
<td>2.35</td>
<td>0.388</td>
<td>0.333</td>
<td>11.0*</td>
<td>46-270</td>
<td>50-140</td>
</tr>
<tr>
<td>Poly-α1CB6</td>
<td>4.29</td>
<td>1.20</td>
<td>0.29</td>
<td>0.49</td>
<td>12.8</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>High-molecular-weight</td>
<td>2.6</td>
<td>2.9</td>
<td>1.2</td>
<td>12.8</td>
<td>4.6-12.6</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>poly-α1CB6†</td>
<td>3.0</td>
<td>3.8</td>
<td>1.2</td>
<td>13.8</td>
<td>4.6-12.6</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

* From Siegel & Monty (1966).
† Separated by gel filtration in buffer 1 (see text).

Fig. 5. Determination of maximum and minimum molecular weights of poly-α1CB6 by gel filtration
Gel filtration on Sepharose 4B (60 cm × 1 cm) in buffer 1. − − − − − , Elution profile for type-I collagen chains and α2CB3.5 in buffer 1. See the Materials and Methods section for experimental details.
seen, the only carbohydrates present in any fraction were galactose and glucose, as initially found in whole poly-α1CB6. 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/KB3H4-labelled CNBr pep-
Table 5. Amino acid and carbohydrate composition of poly-\(\alpha\)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.

<table>
<thead>
<tr>
<th>Amino acid</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp</td>
<td>79.1</td>
<td>80.8</td>
<td>81.8</td>
<td>78.2</td>
<td>80.6</td>
</tr>
<tr>
<td>Asp</td>
<td>51.2</td>
<td>52.6</td>
<td>51.4</td>
<td>49.2</td>
<td>50.0</td>
</tr>
<tr>
<td>Thr</td>
<td>16.6</td>
<td>16.5</td>
<td>16.1</td>
<td>16.5</td>
<td>15.8</td>
</tr>
<tr>
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<td>38.9</td>
<td>40.1</td>
<td>39.1</td>
<td>38.0</td>
</tr>
<tr>
<td>Hse</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glu</td>
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<td>65.8</td>
<td>70.8</td>
<td>69.2</td>
<td>71.0</td>
</tr>
<tr>
<td>Pro</td>
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<td>153.8</td>
<td>140.2</td>
<td>131.8</td>
<td>138.7</td>
</tr>
<tr>
<td>Gly</td>
<td>337.0</td>
<td>319.5</td>
<td>337.4</td>
<td>339.8</td>
<td>344.0</td>
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<td>104.5</td>
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<td>14.9</td>
<td>14.6</td>
<td>15.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Leu</td>
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<td>28.1</td>
<td>26.1</td>
<td>27.5</td>
<td>25.0</td>
</tr>
<tr>
<td>Tyr</td>
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<td>5.6</td>
<td>5.2</td>
<td>5.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Phe</td>
<td>13.3</td>
<td>14.6</td>
<td>12.0</td>
<td>13.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Hyl</td>
<td>8.3</td>
<td>7.1</td>
<td>7.7</td>
<td>8.4</td>
<td>8.2</td>
</tr>
<tr>
<td>Lys</td>
<td>20.7</td>
<td>20.4</td>
<td>19.4</td>
<td>21.7</td>
<td>20.8</td>
</tr>
<tr>
<td>His</td>
<td>5.6</td>
<td>7.6</td>
<td>7.0</td>
<td>7.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Arg</td>
<td>48.9</td>
<td>50.8</td>
<td>50.0</td>
<td>51.4</td>
<td>46.1</td>
</tr>
</tbody>
</table>

| 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-\(\alpha\)1CB6

Poly-\(\alpha\)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 ± 0.02% by wt. of poly-\(\alpha\)1CB6 in these preparations. The lipid content of poly-\(\alpha\)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-\(\alpha\)1CB6 than in the whole CNBr digest. Even higher proportions of this fatty acid were seen in poly-\(\alpha\)1CB6 prepared from acid/urea-washed tendon.

Discussion

Poly-\(\alpha\)1CB6 exhibited unusual and anomalous characteristics on gel filtration. Although eluting

tides from pepsin-treated collagen. As can be seen, \(\alpha\)1CB6 contains a significant proportion of the label. This was shown to be hydroxylysine-galactose by analytical techniques.
Table 6. Lipid content of poly-α1CB6
Poly-α1CB6 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-α1CB6 from unwashed tendon; sample 3. poly-α1CB6 from unwashed tendon (second preparation); sample 4. poly-α1CB6 from acid/urea washed tendon. See text for details.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Myristate (C14)</th>
<th>Palmitate (C16)</th>
<th>Palmitoleate (C16:1)</th>
<th>Stearate (C18)</th>
<th>Oleate (C18:1)</th>
<th>Linoleate (C18:2)</th>
<th>Others</th>
<th>Total % of total analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6</td>
<td>16.8</td>
<td>10.0</td>
<td>3.8</td>
<td>64.4</td>
<td>1.2</td>
<td>2.2</td>
<td>1.77</td>
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<tr>
<td>2</td>
<td>2.2</td>
<td>21.7</td>
<td>7.8</td>
<td>6.7</td>
<td>56.4</td>
<td>0.9</td>
<td>4.3</td>
<td>0.62</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>27.1</td>
<td>8.9</td>
<td>5.8</td>
<td>50.2</td>
<td>1.0</td>
<td>3.5</td>
<td>0.58</td>
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<tr>
<td>4</td>
<td>1.8</td>
<td>19.1</td>
<td>7.4</td>
<td>9.5</td>
<td>58.0</td>
<td>1.2</td>
<td>3.0</td>
<td>0.11</td>
</tr>
</tbody>
</table>

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-α1CB6 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 4 M-urea (Fig. 2a) it exhibited the molecular weight range and elution pattern expected from its behaviour on Sepharose 4B, although in 1 M-CaCl₂ (Fig. 1a) or 1 M-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-α1CB6 changed such that at extremes of pH more poly-α1CB6 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-α1CB6 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-α1CB6 at the A-1.5m void volume were optimal at 50 mg/ml and were decreased at concentrations lower than this (Table 1). This unusual phenomenon proved a useful tool in the purification of poly-α1CB6.

We obtained yields of up to 70% of the expected theoretical quantities of poly-α1CB6 using the modified method reported here and the poly-α1CB6 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-α1CB6, 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-α1CB6 and the correlations obtained were good. The molecular weight ranges covered by the poly-α1CB6 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-α1CB6 molecular species span a mol.wt. range of approx. 50000–350000.

By separating the various molecular weight fractions of poly-α1CB6 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-α1CB6. Polymers in the mol.wt. range 115000–350000, however, accounted for 72% of the material. The amino acid compositions of all polymers of poly-α1CB6 were the same (within the limits of experimental error) showing the material to be chemically homogeneous (Table 5).

Poly-α1CB6 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

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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-α1CB6 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-α1CB6 gave exactly the same result on SDS/polyacrylamide-gel electrophoresis as non-delipidated samples (not shown).

Our data suggest (see below) that poly-α1CB6 contains a mixture of α1CB6 and α1CB5, probably in a ratio of 2:1 or 3:1. Thus the observed micro-heterogeneity of poly-α1CB6 on SDS/polyacrylamide gels may be explained by the number of possible associations of each α1CB6 dimer, trimer, tetramer, etc. with α1CB5. Even greater diversity in the type and size of α1CB6 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 α1CB6 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-α1CB6 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-α1CB6 was prepared from washed and unwashed tendon. A 3-fold increase in stearate content was seen when the fatty acids present in pure poly-α1CB6 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-α1CB6 (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 α1CB6, α1CB5 and α1CB1 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-α1CB6 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-α1CB6 with a 1:1 co-polymer of α1CB6 and α1CB5 (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-α1CB6 to be a 1:1 co-polymer of α1CB6 and α1CB1 (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 (α1CB6)_2-(α1CB5)_1 and (α1CB6)_2-(α1CB5)_2 showed the best fit with the amino acid composition of poly-α1CB6. As increasing amounts of α1CB1 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 α1CB6, α1CB5 and α1CB1, 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-α1CB6.

Under these rigorous comparative conditions the (α1CB6)_3-(α1CB5)_2 and (α1CB6)_2-(α1CB5)_1 co-polymers showed an impressive similarity to the amino-acid content of poly-α1CB6, 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 (α1CB6)_2-(α1CB5)_1, which has a mol. wt. of approx. 43400. This figure is in good agreement with the lowest molecular weight material observed in the poly-α1CB6 preparations.

Whole poly-α1CB6 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 44000–78000 and 78000–115000 respectively). We know that α1CB5 contains one residue each of galactose and glucose (Butler, 1969) and that α1CB6 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 (α1CB6)_2-(α1CB5)_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-α1CB6 showed a galactose/glucose ratio of 4:1 and 5:1, indicating higher proportions of α1CB6 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 (α1CB6) 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 α1CB6 in a molecule in an adjacent penta-fibril coming into reaction distance with a primary α1CB6 containing a reducible cross-link in a separate penta-fibril has to be fulfilled in order to explain the postulated (α1CB6)2-(α1CB5), monomer. This reaction must produce a non-reducible stable bond in order to account for the accumulation of poly-α1CB6 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-α1CB6 prepared has a mol.wt. of between 115000–350000 (Table 2). From five to seventeen α1CB6 peptides must be involved in forming such oligomers, i.e. three to eight (α1CB6)2-(α1CB5) units. When each stable bond, linking two penta-fibrils, is formed, a single α1CB5 must also become covalently associated with the α1CB6 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 α1CB6 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.

We would like to thank Dr. M. Creeth who carried out the initial analytical centrifugation studies, Professor J. Clamp who carried out the carbohydrate analyses, Dr. M. B. Enser who carried out the lipid analyses and Dr. V. C. Duance for helpful and constructive discussions. The work was supported in part by the Nuffield Foundation.

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