# CHANGES IN CROSSLINKING DURING AGING IN BOVINE TENDON COLLAGEN

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## 1. Introduction

Earlier studies on crosslinked peptides in collagen have relied predominantly on the chromatographic preparation of peptides from cyanogen bromide digests of insoluble or borohydride reduced collagen (reviewed [1–3]). The attendant problems of assurance of peptide homogeneity and correct identification and analytical assessment of peptide fragments have hampered much of the work but considerable supportive evidence for the crosslinked peptides deduced from the quarter-stagger overlap model [4] has been obtained. Although little conclusive evidence has been elucidated, the work in [5–7] has established the role of N-terminal peptides in crosslink formation and that in [8] the involvement of C-terminal peptides. The existence of helical–helical intermolecular crosslinks has also been proposed. The possibility of such interactions in hard tissue collagen from work with dentine has been indicated [9,10].

These analyses are tedious and time-consuming and generally have not permitted the comparison of crosslinking in type I collagen from various sources or age groups nor has a study of the relative importance of N-, C-terminal and helix–helix interactions been undertaken. We have described [11] a rapid method, utilising sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, for the direct comparison and quantitation of crosslinking peptides in collagen. Using this technique combined with specific labelling of collagen peptides we now report significant molecular changes during the maturation of bovine tendon collagen that could account for increased stability and possibly lead to the identification of the mature crosslink.

## 2. Materials and methods

### 2.1. *Preparation and labelling of cyanogen bromide peptides*

Bovine achilles tendons were obtained from foetal calf, 6-month calf, and 15-month and 10-year old animals After dissecting away excess fatty tissue the tendons were finely chopped and extracted sequentially with 100 vol. 8 M urea and 0.5 M acetic acid for 4 days at 4°C. The samples were then freeze dried prior to cyanogen bromide digestion for 4 h at 25°C with a dry weight of tendon equivalent to 10 mg/ml in 70% formic acid containing 10 mg/ml cyanogen bromide. After digestion each sample was diluted 10-fold in water and rotary evaporated prior to freeze drying. Dried samples were stored at −20°C.

For comparison of peptides containing reducible components each tendon sample was washed sequentially in phosphate buffer saline (pH 7.4) and water for several days at 4°C. The tendons were then reduced at pH 5.5 with tritiated borohydride as in [12]. Reduced samples were washed, freeze dried and cyanogen bromide digested as above.

A portion of each dried sample was taken up in phosphate-buffered saline (pH 7 4) and tyrosine-containing peptides were labelled with $^{125}I$ by the method in [13]. After labelling, the peptide mixture was exhaustively dialysed against 0.5 M acetic acid, then freeze dried.

### 2.2. *SDS–polyacrylamide gel electrophoresis*

Each of the samples was taken up in SDS buffer [14] and the cyanogen bromide peptides were separated by SDS–polyacrylamide gel electrophoresis in a 2 mm thick slab gel containing an upper stacking gel (5%, w/v, acrylamide) and a lower separating gel (10%, w/v, acrylamide) as in [15].

Gels were routinely stained with Coomassie blue and destained as detailed [14] prior to photography and scanning in a Joyce Chromoscan. Protein content in each band was measured semi-quantitatively by excision of each scanned peak from the paper trace and weighing.

Location of $^{125}$I-labelled peptides was achieved by autoradiography of the dried stained gels and of $KB^3H_4$-labelled peptides by fluorography as in [16]. $\alpha_1$CB6 was prepared from SDS–polyacrylamide gels as follows. From a 5 mg/ml solution of reduced foetal calf tendon cyanogen bromide peptides, 0.6 ml was loaded onto a preparative slab gel. After electrophoresis 2 thin longitudinal side slices and 1 centre slice were taken from the gel and stained in order to locate $\alpha_1$CB6. The appropriate area of gel was then excised and the peptide was eluted as in [17]. Four such gels were necessary for each preparation.

## 3. Results

### 3.1. *Electrophoretic separation of cyanogen bromide peptides*

The electrophoretic mobilities of the major cyanogen bromide (CB) peptides of neutral salt-soluble type I collagen were established using standard peptides prepared by CMC chromatography [18] ($\alpha_1$CB8, $\alpha_1$CB7, $\alpha_1$CB6, $\alpha_1$CB3) and by phosphocellulose chromatography [19] ($\alpha_2$CB4 and $\alpha_2$CB3,5). As can be seen (fig.1) on this gel system $\alpha_1$CB8 ran with an anomalous mobility with respect to the molecular weight of other $\alpha_1$CB peptides. $\alpha_1$CB6 ran as a doublet with the slower-moving band corresponding to the correct molecular weight for this peptide ($\alpha_1$CB6a) and the faster-moving band exhibiting a molecular weight characteristic of $\alpha_1$CB6b [18]. The latter peptide appeared to have a molecular weight consistent with the loss of 10–15 amino acids from $\alpha_1$CB6a.

The identification of the doublet as $\alpha_1$CB6 was

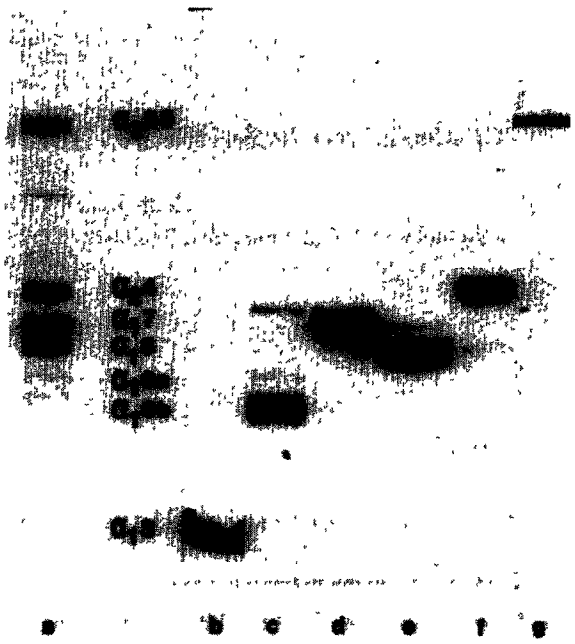

Fig.1a SDS–polyacrylamide gel electrophoresis of CB peptides prepared as standards from neutral salt-soluble collagen: (a) Neutral salt soluble collagen CB digest, (b) $\alpha_1$ CB3; (c) $\alpha_1$ CB6; (d) $\alpha_1$ CB8; (e) $\alpha_1$ CB7, (f) $\alpha_2$CB4; (g) $\alpha_2$ CB3,5. Samples (b–e) were prepared by CMC chromatography [18] and (f,g) by phosphocellulose chromatography [19].

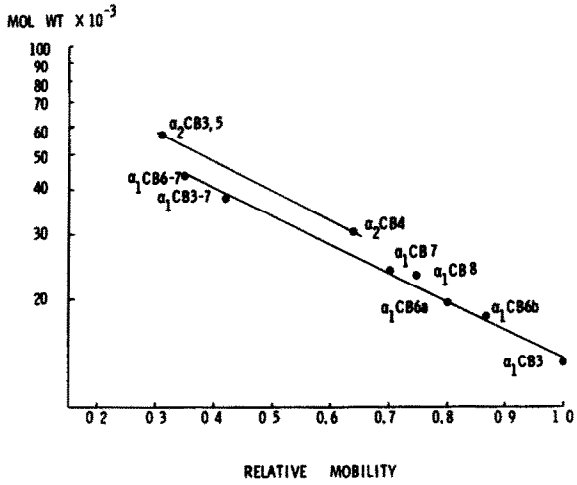


Fig.1b. Calibration curve for major CB peptides of neutral salt-soluble type I collagen on SDS–polyacrylamide gels.

further confirmed by excision of the band from preparative gels of reduced foetal bovine tendon CB peptide and subsequent amino acid analysis of the peptide. The amino acid content was consistent with an approximate 1.1 mixture of $\alpha_1$CB6a and $\alpha_1$CB6b and the peptides did not contain any homoserine or reduced aldimine crosslinks. Although the doublet in foetal bovine tendon ran slightly slower than the same peptide from neutral salt soluble foetal bovine skin type I, it comigrated with the same doublet from neutral salt-soluble calf tendon type I (fig.2f,g). This probably reflects differences in glycosylation in $\alpha_1$CB6 from skin and tendon.

### 3.2. *Changes on aging in cyanogen bromide peptides of bovine tendon collagen*

Figure 2 shows an SDS–polyacrylamide gel of the CB peptides obtained from neutral salt soluble type I collagen, foetal calf achilles tendon, 6 m calf achilles tendon, 15 m bovine achilles tendon and 10 year bovine achilles tendon. The $\alpha_1$CB6 doublet disappeared on aging and was not detectable in the 10 year sample. Table 1 illustrates this point by quantitation of the bands from scans of the gels. Examination of the fluorographs of $KB^3H_4$-reduced tendon shown in fig.3a demonstrated that $\alpha_1$CB6 occurred as a doublet comigrating with $\alpha_1$CB7 and $\alpha_1$CB8 in both foetal and neonatal samples. However, this band was neglible in the 10 year sample and the majority of label was associated with material which did not enter the gel.

The characteristics of the $\alpha_2$CB3,5 band were different in the 10 year sample compared to the same

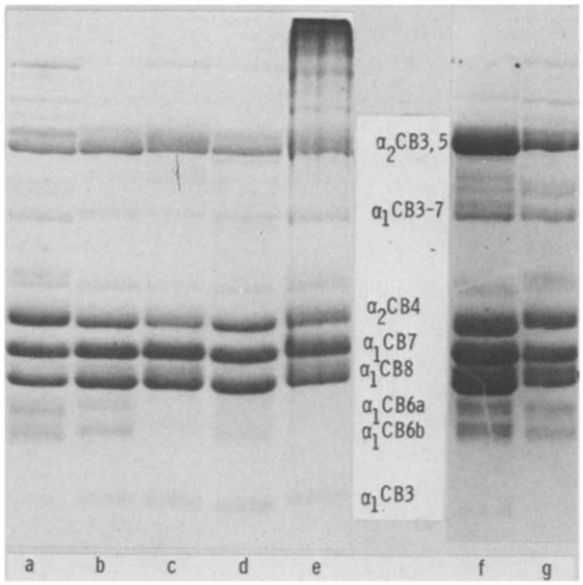


Fig.2. SDS–polyacrylamide gel of CB peptides derived from. (a) Neutral salt soluble calf skin type I, (b) foetal bovine tendon, (c) 6-month calf tendon; (d) 2-year bovine tendon; (e) 10-year bovine tendon, (f) as (b); (g) neutral salt-soluble foetal bovine tendon type I.

Table 1
Quantitation of the major peptides relative to $\alpha_2$CB4 in bovine achilles tendon CB digests from animals of differing ages

| Source of material | Peptide | | | | | | |
|---|---|---|---|---|---|---|---|
| | $\alpha_1$CB3 | $\alpha_1$CB6b | $\alpha_1$CB6a | $\alpha_1$CB7 | $\alpha_1$CB8 | $\alpha_2$CB4 | $\alpha_2$CB3,5 |
| Neutral salt-soluble type I | 0 3 | 0.61 (10 ) | 0.55 (9) | 0.90 (15) | 0.68 (11) | 1 0 (16) | 2.42 (39) |
| Foetal calf achilles tendon | n.d. | 0 35 ( 6 ) | 0 24 (4) | 0.90 (16) | 0.76 (13) | 1.0 (17) | 2.47 (44) |
| 6 m calf achilles tendon | n.d. | 0 19 ( 3 ) | 0.09 (2) | 1.29 (19) | 1.02 (15) | 1 0 (15) | 3.12 (46) |
| 15 m bovine achilles tendon | n.d | 0.16 ( 2.5) | 0 0 (0) | 1.10 (20) | 0 83 (16) | 1 0 (17) | 2 24 (44) |
| 10 year bovine achilles tendon | 0 26 | 0 0 ( 0 ) | 0 0 (0) | 1.22 (21) | 0 96 (17) | 1.0 (18) | 2.44 (43) |

n.d , not done

Figures in parentheses denote % of total protein in the 6 major peptides

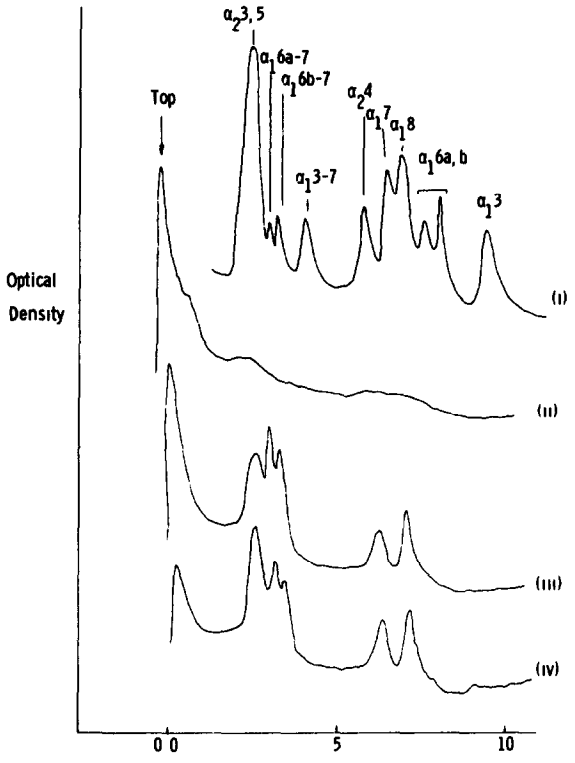


Fig 3a. SDS–polyacrylamide gel electrophoresis of $KB^3H_4$-labelled CB peptides from bovine tendon from animals of various ages. Scans of (i) foetal tendon CB peptides stained for protein and fluorographs of (ii) 10 year bovine tendon CB peptides, (iii) 6 m bovine tendon CB peptides, (iv) foetal bovine tendon CB peptides.

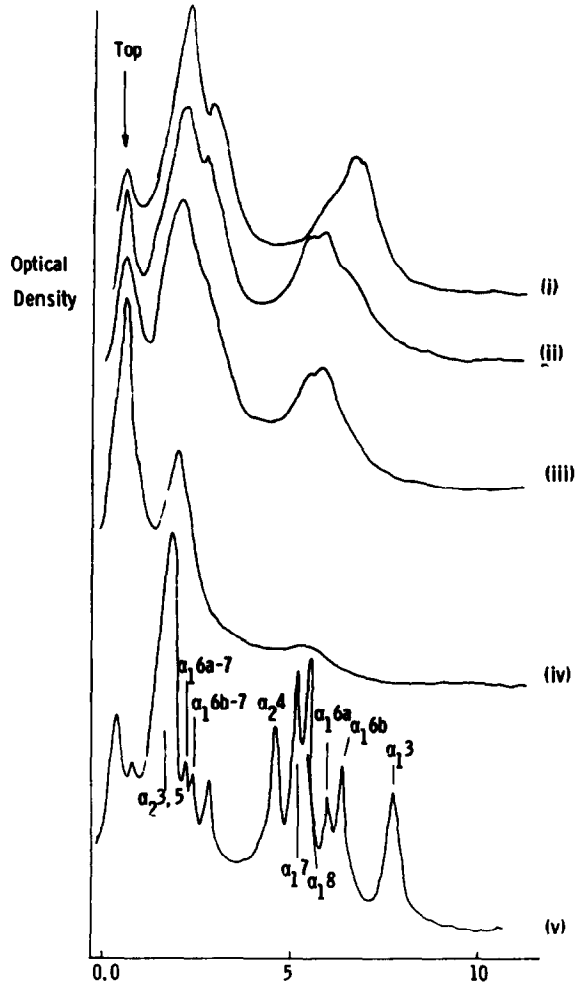


Fig.3b SDS–polyacrylamide gel electrophoresis of $^{125}I$-labelled CB peptides from bovine tendon from animals of various ages Scans of autoradiographs of. (i) foetal tendon CB peptides, (ii) 6 m calf tendon CB peptides, (iii) 15 m bovine tendon CB peptides, (iv) 10 year bovine tendon CB peptides; (v) foetal tendon stained for protein

peptide in neutral salt soluble collagen. The band lost its sharp front and ran with a slightly slower mobility corresponding to a molecular weight increase of about 2000–4500. The peptide appeared to be cross-linked via a reducible bond to a small peptide as it incorporated radioactivity on reduction with $KB^3H_4$. This result is consistent with a similar observation reported in corneal type I $\alpha_2$CB3,5 [20] but the importance of this crosslink in stabilising the collagen matrix is not clear.

### 3.3 *Labelling of tyrosine containing peptides with* $^{125}I$

Figure 3b shows scans of autoradiographs of $^{125}I$-labelled cyanogen bromide peptides separated by SDS–polyacrylamide gel electrophoresis. In the foetal sample only $\alpha_1$CB6 and $\alpha_2$CB3,5 exhibited any radioactivity. In the 6 m sample most of the label in the $\alpha_1$CB6 region disappeared and moved to a molecular weight in the region of $\alpha_1$CB7 and $\alpha_1$ CB8. Concomitantly, the amount of labelled material which did not enter the gel increased. In the 15 m sample all the label in $\alpha_1$CB6 disappeared and co-migrated with $\alpha_1$CB7 and $\alpha_1$CB8. In the 10 year sample the majority of the label did not enter the gel In all the samples the amount of label in $\alpha_2$CB3,5 remained virtually

constant, but the amount of label in $\alpha_1$CB6-7 region gradually decreased with age.

## 4. Discussion

When young tissue was reduced with $KB^3H_4$ the majority of label was associated with peptides comigrating with $\alpha_1$CB7 and $\alpha_1$CB8 and $\alpha_2$CB3,5. The 2 former comigrating peptides represent $\alpha_1$CB6a and $\alpha_1$CB6b crosslinked via a reduced aldimine bond to another small peptide, either $\alpha_1$CB1 or $\alpha_1$CB5 [5,8]. However, the 10 year sample showed incorporation of label only into peptides which did not enter the polyacrylamide gel matrix. A similar result was obtained when peptides were labelled with $^{125}I$. Only $\alpha_1$CB6 and $\alpha_2$CB3,5 labelled in this case as the only other collagen peptides containing tyrosine, $\alpha_1$CB0,1 and $\alpha_2$CB1, should elute from the bottom of the gel or will be involved in reducible crosslinks with $\alpha_1$CB6 [5] Again, the only labelled material seen in the 10 year sample, except for $\alpha_2$CB3,5, was high molecular weight peptide which did not enter the gel.

Consideration of these results suggests that the mechanism of crosslinking in bovine tendon involves the production in foetal and neonatal tissue of a crosslinked peptide involving $\alpha_1$CB6 and having a similar molecular weight to that of $\alpha_1$CB7. This crosslink is then stabilised by the incorporation of the peptide into high molecular weight material We can conclude then, that the stable crosslink must be multivalent, linking several $\alpha$ chains. This fits with our knowledge of the reducible crosslinks which are stable under physiological conditions. Only addition of extra $\alpha$ chains across this aldimine link could lead to the further stabilisation of the collagen matrix observed in aging. The question arising from this interpretation is which peptide or peptides are involved in the formation of complex crosslinks with crosslinked $\alpha_1$CB6? The major peptides show little or no change in their relative amounts during aging when quantitated relative to $\alpha_2$CB4 (table 1). Similarly, no significant change in the amounts of these peptides could be seen when quantitated as % total protein. Thus, none of the major helical peptides can be involved in the formation of reducible crosslinks or complex crosslinks during aging.

Within the parameters of the quarter-stagger overlap theory it is possible to postulate the regular linear alignment of pentafibrils within the fibre such that, along each edge, the triple helices are in non-staggered array with those of the adjacent pentafibril. In such a structure it would be possible for adjacent C-termini to interact forming stable crosslinks from fibril to fibril both horizontally and vertically through the fibre. Such a process may indeed be expected to lead to the kind of stabilisation of the collagen fibre that occurs during maturation, and to the observed production of $(\alpha_1$CB6-X,Y$)_n$ polymers in cyanogen bromide digests of such material, where X and Y are small peptides such as $\alpha_1$CB0,1 and $\alpha_1$CB5.

## Acknowledgement

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