Collagen heterogeneity and quantification in developing bovine nuchal ligament

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The collagenous components were investigated in peptic digests of developing bovine nuchal ligament. Types I and III collagen were the major species isolated, but the presence of types IV, V and VI was also shown. Changes in the pepsin-susceptibility of nuchal ligament during foetal development were observed. CNBr-cleavage peptide analysis indicated that type I collagen became cross-linked rapidly, as evidenced by the lack of $\alpha 1(I)CB6$. At present it is not clear if this decrease in pepsin-susceptibility is due to cross-linking of collagen, to increased deposition of elastin, or to both. Quantification of collagen types I and III was shown to depend on the method used. When pepsin-solubilized material was examined an apparent increase in type III collagen with respect to foetal age was observed, whereas when CNBr digests of intact ligament were examined a relatively constant amount of type III collagen (approx. 24%) was found. The constant amount of type III collagen observed during foetal development changed at birth and increased in mature nuchal ligament to represent approx. 45% of the total collagen.

The developing nuchal ligament of grazing animals has provided a useful model system for studies on elastic-fibre development (Cleary *et al.*, 1967; Wirtschafter *et al.*, 1967; Jones *et al.*, 1980; Davidson *et al.*, 1982). Dramatic changes in elastin deposition are seen in this tissue in the latter half of gestation, and accordingly it has been used in detailed studies on the regulation of elastin production (Mecham *et al.*, 1981; Mecham, 1981; Davidson *et al.*, 1982) and on the synthesis of the glycoprotein components of elastic fibres (Sear *et al.*, 1978, 1981a,b; Knight *et al.*, 1984). In all these studies the presence of collagen has been noted, but the nature of the major collagens present in this classical elastic tissue have not been previously examined.

Several genetically distinct collagen molecules have now been characterized and the tissue distributions of the various collagen types determined. Collagen types I and III are the major collagens in elastic tissues such as aorta, lung (Epstein & Munderloh, 1975), uterus (Abedin *et al.*, 1982) and skin (Epstein, 1974), and evidence for the presence of types IV and V in such tissues has also been reported (for review see Weiss & Ayad, 1982).

More recently a novel microfibrillar form of collagen, originally called HMW aggregate and intimal collagen, but now designated type VI collagen, has been identified as a minor component of a number of tissues (Chung *et al.*, 1976; Laurain *et al.*, 1980; Jander *et al.*, 1983; Odermatt *et al.*, 1983).

Type III collagen is always found in tissues in association with type I, but the relative proportions of types I and III collagens vary from tissue to tissue. In some cases a preponderance of type III to type I collagen has been demonstrated in foetal tissues, and during development there is a fall in the relative proportion of type III (Epstein, 1974). In other situations large amounts of type III collagen have been found in adult tissues (Hansen & Bentley, 1983; van Amerongen *et al.*, 1983). The significance of collagen types with respect to both physical properties and tissue function is not clearly understood, although current evidence suggests that type I affords tensile strength, whereas type III is found in tissues that experience 'mechanical' stress resulting in distension (for review see Miller, 1976). Type III collagen has a smaller fibril diameter than type I, and would therefore appear to be more suited to function in tissues undergoing deformation. In this respect type III collagen provides a certain measure of elasticity in soft tissues that endure compressive forces but

Abbreviation used: SDS, sodium dodecyl sulphate.

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possess little elastin, such as dental pulp (Shuttleworth *et al.*, 1978) and periodontal ligament (Limeback *et al.*, 1978). Although it is generally found that the amount of type III collagen is high in elastin-rich tissues, the biological significance of its apparent predominance in elastic tissues and its inter-relationship with elastin are not understood.

In the present study we have used the bovine nuchal ligament as a model system in which to study the collagens of a typical elastic tissue during development. Particular emphasis has been placed on studying the relationship between collagen types I and III and their possible role in connective tissues.

Materials and methods

Materials

Bovine foetuses were obtained from Manchester Abattoir within 2 h of maternal death and foetal age was assessed from the crown-rump length (Bogart, 1959). Nuchal ligaments were dissected and freed from surrounding tissue and powdered in a stainless-steel mill under liquid N₂.

Tris, SDS (specially purified), glycerol, urea, chloramine-T, 4-dimethylaminobenzaldehyde and HClO₄ were purchased from Fisons Scientific Apparatus (Loughborough, Leics., U.K.). Bromophenol Blue, ammonium persulphate, *NNN'*-tetramethylethylenediamine and periodic acid/Schiff reagent were supplied by BDH Chemicals (Poole, Dorset, U.K.). Pepsin (EC 3.4.23.1), glycine, phenylmethanesulphonyl fluoride, acrylamide, *NN'*-methylenebisacrylamide, Coomassie Brilliant Blue R, 2-mercaptoethanol, CNBr and dithiothreitol were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). DEAE-cellulose (DE52) was purchased from Whatman Chromatography Services (Wirral, Merseyside, U.K.).

Isolation and fractionation of collagens

Powdered tissue samples were extracted twice with 50 mM-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl (0.5–1 mg wet wt. of tissue/ml, at 4°C for 24 h) and twice with 0.5 M-acetic acid (0.5–1 mg wet wt. of tissue/ml, at 4°C for 24 h). Insoluble material was removed at each step by centrifugation (40000g for 1 h) at 4°C. Appropriate fractions were pooled to give, respectively, a salt-soluble extract, an acid-soluble extract and a residue. The residue was suspended in 0.5 M-acetic acid, pH 2.5, and digested with pepsin (10–20 mg/g wet wt. of tissue) at 4°C for 48 h. Pepsin-solubilized material was fractionated (Scheme 1) in order to isolate the major collagen species present (Chung & Miller, 1974; Abedin *et al.*, 1982). The isolated collagen types I, III and V were characterized by subunit

composition on SDS/polyacrylamide-gel electrophoresis, by CNBr-cleavage peptide analysis and by amino acid analysis.

CNBr cleavage

Purified collagen samples (1–2 mg dry wt.) were digested with CNBr as described by Scott & Veis (1976) and analysed by SDS/polyacrylamide-gel electrophoresis. In experiments where intact tissue was to be analysed, samples of powdered tissue (approx. 100 mg) were homogenized by using the method described by Laurent *et al.* (1981) and then digested with CNBr as indicated above. Undigested material was separated and washed on a filter paper with water. The supernatant and washings were pooled and freeze-dried and the proportion of collagen types I and III was determined by SDS/polyacrylamide-gel electrophoresis on 15% gels. The residue was analysed for amino acid composition.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis on 6% gels (for collagen subunit composition analysis) or 15% gels (for CNBr-cleavage peptide analysis) was achieved by following the method of Laemmli (1970) except that a final concentration of 0.5 M-urea was included in the electrode chamber buffer and in both the stacking and main gels, to improve resolution.

Quantification of type III collagen

In pepsin digests of ligament the presence of $\alpha 1(\text{III})$ chains was assessed by using the interrupted reduction method described by Sykes *et al.* (1976).

In intact ligament CNBr-cleavage peptides were analysed by SDS/polyacrylamide-gel electrophoresis. Peaks were identified by comparison with authentic CNBr-cleavage peptides of types I and III collagen and quantified by reference to standard mixtures of peptides of types I and III in the ratios 9:1, 3:1 and 1:1. Coomassie Blue-stained gels were scanned on a Beckman model 25 spectrophotometer.

Amino acid analysis

Samples were hydrolysed (1 mg/2 ml of redistilled 6 M-HCl) for 18 h at 110°C in sealed evacuated test tubes. Excess HCl was removed by rotary evaporation at 40°C, and the sample was analysed on a JEOL JLC 6AH amino acid analyser.

Hydroxyproline assays

The hydroxyproline content of various fractions was determined after hydrolysis in 6 M-HCl at 110°C for 24 h by the method of Stegeman & Stadler (1967).

Scheme 1. Fractionation of pepsin-solubilized material

Ion-exchange chromatography

Samples (10–60mg) were dissolved in 50mM-Tris/HCl buffer, pH 7.5, and applied to a column (1.6cm × 10cm) of DEAE-cellulose that had been pre-equilibrated with this buffer. Each sample was washed on to the column with 100ml of starting buffer, then a linear gradient (0–500mM-NaCl) was applied over 200ml. Fractions (5ml) were collected at a flow rate of 50ml/h.

Results

Extractability with pepsin

Analyses showed that less than 2% of the total hydroxyproline was soluble in the neutral salt and acetic acid extracts of nuchal ligament at different stages of foetal development. The proportion of hydroxyproline solubilized by pepsin as a function of size of foetus from which the nuchal ligament was taken is shown in Fig. 1. With increasing foetal age there was an apparent decrease in the proportion of hydroxyproline extracted, from approx. 55% of the total hydroxyproline solubilized in young embryos to approx. 35% in later foetal tissue.

The pepsin-solubilized collagens were separated as described in Scheme 1, and the distribution of collagen in the various fractions was assessed by hydroxyproline determination (Table 1).

Fig. 1. Percentage of total hydroxyproline extracted by pepsin from nuchal ligaments at different stages of foetal development

The slope of the line was calculated by using best fit regression analysis. The 95% confidence limits are shown (----). At term foetal calves are approx. 95cm from head to rump.

Collagen types I, III and V separated by these procedures were characterized by subunit composition on SDS/polyacrylamide gels, by CNBr-cleavage peptide analysis and by amino acid analysis. These analyses indicated that while type I collagen was free of type III, the type III preparation contained trace amounts of type I. Electrophoretic analysis of the type V collagen fraction revealed the presence of $\alpha 1(V)$, $\alpha 2(V)$ and $\alpha 3(V)$ collagen chains which were not reduced by mercaptoethanol. Amino acid analyses of the separated collagens were all consistent with previously published analyses (Chung & Miller, 1974; Abedin *et al.*, 1982).

A minute amount of material (0.1–0.4% of the total pepsin-solubilized hydroxyproline) with the solubility characteristics of type IV collagen was isolated (Scheme 1). Gel electrophoretic analyses of this material before and after reduction confirmed its identity as type IV collagen (gels not shown). A fraction with the solubility characteristics of type VI collagen was also isolated and identified by gel electrophoresis. This fraction represented approx. 1% of the total solubilized collagen and was used as a type VI collagen marker in other studies (Knight *et al.*, 1984).

Peptides obtained by CNBr cleavage of purified ligament collagen types I, III and V were examined on 15% disc gels. Densitometric scans of Coomassie Blue-stained gels of peptides from collagen types I and III are shown in Figs. 2(a) and 2(c) respectively. The peptide patterns obtained compare with previously characterized collagen types I and III (Weber *et al.*, 1977) and peptide peaks specific to each collagen were identified. The type V collagen peptide pattern (results not shown) was comparable with that for previously characterized type V collagen (Sage *et al.*, 1980), and was distinct from patterns obtained for collagen types I and III.

Quantification of type III collagen in intact ligament

The amounts of collagen types I and III in samples of intact nuchal ligament were estimated from densitometrically determined amounts of specific CNBr-cleavage peptides. Fig. 2 shows peptide patterns obtained for purified collagen types I and III and for CNBr-digested ligament. Peptides $\alpha 1(I)CB8$ and $\alpha 1(III)CB8$, identified on the basis of their known mobilities (Laurent *et al.*, 1981; Light, 1982), were quantified. Control analyses of CNBr-cleavage peptides demonstrated a linear relationship between the proportions of types I and III and the $\alpha 1(I)CB8:\alpha 1(III)CB8$ ratio. The relative proportions were estimated in this way for a range of foetal sizes, for two calves (1 month old), and for two mature ligaments (Fig. 3). No great change in the proportion of type III was observed during foetal life, but there was a marked

Table 1. *Distribution of hydroxyproline after fractionation of pepsin-solubilized nuchal ligament as a percentage of total pepsin-soluble hydroxyproline*

(a) Material isolated by precipitation with 0.7M-NaCl (pH 2.5) (types I and III). (b) Material isolated by precipitation with 1.2M-NaCl (pH 2.5) (types IV and V). (c) Material remaining soluble after precipitation at 1.2M-NaCl (pH 2.5). See Scheme 1.

Size of foetus (cm)	Distribution (%)		
	(a) 0.7M-NaCl ppt.	(b) 1.2M-NaCl ppt.	(c) 1.2M-NaCl supt.
29	76.6	9.2	14.2
52	80.0	3.0	17.0
56	85.1	2.4	12.5
69	73.5	5.8	20.7
73	67.7	3.9	28.4

increase from 27% at birth to 45% in the mature ligament. A noteworthy feature of the present study was the absence of $\alpha 1(I)CB6$ from CNBr-cleavage peptide patterns obtained for ligaments analysed at all stages of foetal development (Fig. 4).

Quantification of type III collagen in pepsin-solubilized collagens

The relative proportions of collagen types I and III present in the 0.7M-NaCl/acid precipitate was determined by electrophoretic analysis of the α -chains (see the Materials and methods section). The percentage of type III collagen obtained for a range of foetal sizes is presented in Fig. 5 and shows an apparent increase in type III collagen during development.

Identification of elastin in CNBr-digest residues

Amino acid analyses of the residues left after CNBr digestion (Table 2) clearly suggest that all the collagen was solubilized by CNBr treatment and that the residue was composed of elastin. On the basis of hydroxyproline analyses on the CNBr-digest residues it can be calculated that elastin accounted for only 4% of the total (tissue) hydroxyproline in a 37 cm foetus and this value rose to 15% in a 92 cm foetus. This result is consistent with the increase in elastin deposition observed during development (Cleary *et al.*, 1967) but suggests that, even at term, over 80% of the hydroxyproline in the ligament is accounted for by collagen.

Characterization of the polypeptides soluble in the 1.2M-NaCl/acid supernatant

Precipitation of collagens by salt fractionation (Scheme 1) left 12–28% of the pepsin-solubilized hydroxyproline in the 1.2M-NaCl/acid supernatant. Addition of salt to 2M-NaCl precipitated a small quantity of material identified as type VI collagen as described above. However, most of the hydroxyproline remained soluble, and preliminary

electrophoretic analysis of this fraction on 15% SDS/polyacrylamide gels in the presence or in the absence of reducing agent revealed a complex spectrum of small- M_r components. When this 2M-NaCl/acid supernatant was applied to a DEAE-cellulose column the material was resolved into three peaks (Fig. 6).

Electrophoretic analysis of the material in peak A (Fig. 6) showed only a diffuse Coomassie Blue-stained band running just behind the dye front. The amino acid composition of peak A (Table 3) showed some similarities to that of elastin (see Table 2) and to that of a pepsin-solubilized form of elastin (Trelstad, 1974). It was therefore concluded that peak A was composed mainly of peptides derived from elastin.

Characterization of peak B by SDS/polyacrylamide-gel electrophoresis revealed a range of small- M_r polypeptides. Analysis of reduced and non-reduced samples showed that some of the polypeptides were disulphide-bonded. Periodic acid/Schiff-stained gels identified a single glycopeptide (M_r 20000) (Fig. 7, gel 1) that, on reduction, yielded four smaller- M_r species (Fig. 7, gel 2). Amino acid analysis of peak B (Table 3) indicated that the peptides were essentially non-collagenous, but the presence of a very high content of cysteine is consistent with the high degree of disulphide-bonding detected between these peptides.

Electrophoretic analysis of peak C revealed a single major Coomassie Blue-stained band of M_r 30000 that, on reduction, ran as two bands of slightly larger M_r . When a sample of the pepsin used in this investigation was analysed by an identical procedure, a very large peak was obtained in the position of peak C, and SDS/polyacrylamide-gel electrophoresis analysis confirmed that this peak was indeed pepsin. These observations and the presence of hydroxyproline in peak C (Table 3), are consistent with the report that a hydroxyproline-containing peptide occurs in commercial pepsin preparations (Matsui *et al.*, 1982).

Fig. 2. Densitometric scans of Coomassie Blue-stained disc gels of the CNBr-cleavage peptides obtained by digestion of (a) type I collagen, (b) intact nuchal ligament and (c) type III collagen

Peptides denoted are specific to type I collagen [$\alpha 1(I)CB8$] (1) and to type III collagen [$\alpha 1(III)CB8$] (2); 3 represents the dye front. The direction of migration was from cathode (–) to anode (+).

Table 2. Amino acid compositions for the CNBr-digest residues from a 37 cm foetus and a mature adult nuchal ligament. Comparison is made with previously characterized elastin (Ross & Bornstein, 1969).

Amino acid	Composition (residues/1000 amino acids)		
	Ligament from 37cm foetus	Mature ligament	Elastin
Hyp	17	5	11
Asp	16	5	6
Thr	13	8	9
Ser	14	8	10
Glu	27	14	15
Pro	99	116	120
Gly	299	328	324
Ala	234	229	223
Cys	Trace	6	4
Val	145	146	135
Ile	33	27	26
Leu	72	68	61
Tyr	Trace	8	7
Phe	4	26	30
Hyl	Trace	–	–
Lys	11	3	7
His	3	Trace	1
Arg	8	5	5

Fig. 3. Relationship between percentage of type III collagen found in intact bovine nuchal ligament and size of animal from which the tissue was taken

Percentage type III was estimated by CNBr digestion of intact ligament and determination of the amounts of peptides specific to type I and type III collagen (shown in Fig. 2). $\blacktriangle$ symbols represent the mean values of either two or three determinations and bars represent the range.

Thus fractionation of the material soluble in the 1.2M-NaCl/acid supernatant showed that a small proportion of hydroxyproline could be accounted for by the presence of type VI collagen, some by

Fig. 4. SDS/polyacrylamide-gel electrophoresis of CNBr-cleavage peptides of intact ligament samples from a range of foetal sizes

Tracks 1, 2, 3, 4 and 5 are samples from 31 cm, 69 cm, 53 cm, 51 cm and 75 cm foetuses respectively. Track 6 shows the CNBr-cleavage peptide pattern of type I collagen. The position of $\alpha 1(I)CB6$ is marked with an arrow.

elastin peptides and some from pepsin, and the remainder was associated with a glycopeptide-containing fraction.

Discussion

The biological roles of the genetically distinct collagens are not well understood. However, some insight into the structure-function relationships of the various collagens may be obtained by chemical analyses and immunohistochemical studies, which provide information on the relative proportions and distribution of collagens within specific tissues. This approach depends in large part on accurate quantification of the different collagen

Fig. 5. Relationship between percentage of type III collagen solubilized by pepsin and the size of the foetus from which ligament sample was taken

The percentage of type III collagen solubilized was calculated after interrupted-reduction SDS/polyacrylamide-gel electrophoresis of the 0.7M-NaCl-precipitate fraction (Scheme 1), and the relative amounts of $\alpha 1(I)$ and $\alpha 1(III)$ were assessed after densitometric scanning of Coomassie Blue-stained gels.

Fig. 6. DEAE-cellulose chromatographic profile of the 2M-NaCl/acid supernatant (Scheme 1)

The column (1.5 cm $\times$ 10 cm) was equilibrated with 50M-Tris/HCl buffer, pH 7.5, and fractions (5 ml) were collected at a flow rate of 50 ml/h. A linear salt gradient 0–500 mM over 200 ml was used to elute the retained material. —, A_{280} ; ----, concn. of Cl^- . The bars show the fractions taken for isolation of samples A, B and C.

types and the availability of monospecific antisera. The chemical studies reported in the present paper were undertaken to assess the relative proportions of the major interstitial collagens in the foetal

Fig. 7. SDS/polyacrylamide-gel electrophoresis of peak B (Fig. 6) on 10% gels and stained for carbohydrate with periodic acid/Schiff reagent (Fairbanks *et al.*, 1971). Gel 1, unreduced; gel 2, reduced with mercaptoethanol. Positions of CNBr-cleavage peptide markers are shown: a, $\alpha 1(\text{I})\text{CB8}$ (M_r 24000); b, $\alpha 1(\text{I})\text{CB6}$ (M_r 18000); c, $\alpha 1(\text{I})\text{CB3}$ (M_r 13500).

bovine nuchal ligament and to evaluate any correlation with the changes in elastin content known to occur during development (Cleary *et al.*, 1967). Characterization of pepsin-solubilized collagens was initially undertaken and two methods were then used to quantify the major collagens identified.

Approx. 55% of the nuchal ligament hydroxyproline could be solubilized by pepsin treatment of tissues isolated in the early stages of foetal development but, as term approached, this value fell to approx. 35%. Decreased pepsin solubility of collagen with age may reflect an increase in more stable cross-links between molecules (Light & Bailey, 1980). Interestingly, the peptides obtained

Table 3. Amino acid compositions of peaks A, B and C (Fig. 6) isolated by DEAE-cellulose chromatography of 2M-NaCl supernatant fraction

Amino acid	Composition (residues/1000 amino acids)		
	Peak A	Peak B	Peak C
Hyp	34	13	10
Asp	15	95	122
Thr	15	54	72
Ser	18	47	112
Glu	29	90	94
Pro	116	91	75
Gly	285	142	133
Ala	207	91	109
Cys	11	85	Trace
Val	146	53	62
Met	2	11	Trace
Ile	22	35	62
Leu	38	48	81
Tyr	7	20	9
Phe	24	28	40
Hyl	Trace	Trace	Trace
Lys	20	28	23
His	3	11	4
Arg	10	33	16

from CNBr digestion of ligaments of different foetal ages show no peptide $\alpha 1(\text{I})\text{CB6}$ even in the youngest foetus (31 cm) (Fig. 6). This peptide has been implicated in the formation of stable cross-links through $\alpha 1(\text{I})\text{CB1}$ or $\alpha 1(\text{I})\text{CB5}$ (Light & Bailey, 1979) and its absence in foetal ligament would indicate the formation of stable cross-links at a very early stage. The decrease in collagen solubility with pepsin may also reflect an inaccessibility of collagen to the enzyme because of the vast increase in elastin deposited towards term (Cleary *et al.*, 1967). Such a conclusion would be consistent with the studies of Faris *et al.* (1978) indicating that removal of elastin, prior to collagen extraction, greatly increases collagen solubility in aorta.

Collagen types I, III and V were isolated from ligament samples at all stages of development. The fraction containing collagen types I and III accounted for the majority (68–85%) of the hydroxyproline solubilized by pepsin. A small proportion of hydroxyproline was also recovered in the fractions identified as containing collagen type V (2–9%) and type IV (0.1–0.4%). Immunofluorescence studies using an antibody to type IV collagen on ligament tissue sections showed that the type IV collagen was localized in capillary vessel walls (C. A. Chambers, S. Ayad, C. A. Shuttleworth & M. E. Grant, unpublished work). Although the major collagens solubilized by pepsin are precipitated from acid by 1.2M-NaCl, a considerable propor-

tion of the hydroxyproline remained soluble (Table 1). Increasing the salt concentration to 2M-NaCl enabled the isolation of material with electrophoretic characteristics of the large- M_r aggregate described by Abedin *et al.* (1982), and termed type VI collagen (Odermatt *et al.*, 1983). The presence of type VI collagen and the identification of the $\alpha 3(V)$ chain in pepsin digests of ligament are consistent with an earlier report that these two collagens occur together in the same tissues (Abedin *et al.*, 1982).

In view of the complete solubilization of tissue collagen achieved with CNBr it is probable that the results obtained in this way are a reflection of the true situation in the tissue. The constant proportion of type III collagen observed in foetal nuchal ligament, however, may not apply to other tissues, since previous studies (Epstein, 1974; Sykes *et al.*, 1976) have shown a decrease in this collagen in developing foetal skin. Interestingly, at birth the proportion of type III in the present investigation was shown to rise, and in the mature ligament to represent approx. 45% of the total collagen. These results correlate with the studies by Light (1982), who found an increased proportion of type III collagen relative to type I in ageing human skin.

High concentrations of type III collagen have been measured in such tissues as aortic media (70%) (McCullagh & Balian, 1975); abdominal aorta (42%); aortic arch (37%) (Hansen & Bentley, 1983); and lung (30%) (Laurent *et al.*, 1981; Seyer *et al.*, 1981), where tissue is subjected to greater changes in extensibility than tissue such as adult skin or tendon (Light, 1982; Jimenez *et al.*, 1978). The increased proportion of type III collagen observed with maturity in the present study may therefore reflect the increased functional demands to which an adult nuchal ligament is subjected compared with that of young and foetal tissue.

The use of pepsin to release collagens from the extracellular matrix is based on the proteinase-resistance of the triple-helical portion of collagen molecules. This procedure permits the isolation of the major interstitial collagens from tissues, and in the developing nuchal ligament it appears that collagen types I and III, together with elastin, represent the major structural elements. The roles of type V and type VI collagens in the tissue are not clear. Type V collagen has been found in a pericellular location, and may be an indication of the cellularity of the developing tissue (Gay & Miller, 1978). It has been suggested that the recently characterized type VI collagen is a pepsin-derived fragment originating from microfibrillar components (Furthmayr *et al.*, 1983). Microfibrils are clearly evident in developing nuchal ligament (Cleary *et al.*, 1967) and may be the source of the

type VI collagen tentatively identified in the present study. It is noteworthy that microfibrillar structures in connective tissues are known to comprise one or more glycoproteins (Birembaut *et al.*, 1982) and Sear *et al.* (1978, 1981a,b) have identified in cell cultures of bovine nuchal ligament fibroblasts a partially collagenous glycoprotein that may be related to microfibrils. Evidence that this collagenous glycoprotein is the parent molecule from which type VI collagen is derived is presented in the accompanying paper (Knight *et al.*, 1984). Further studies will be required to ascertain whether the peptides (particularly the glycopeptide) retained by ion-exchange chromatography (fraction B, Fig. 6) are derived from the highly glycosylated and disulphide-bonded type VI collagen precursor.

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