# Extraction and characterization of the tissue forms of collagen types II and IX from bovine vitreous

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We report for the first time that, after centrifugation of adult bovine vitreous, the hyaluronan-rich supernatant contains collagens which can be isolated in their intact forms by precipitation with 4.5 M NaCl. This precipitate constituted approx. 4% of the total vitreous collagen and comprised collagen types IX and II (in the approximate ratio of 4:1) with negligible amounts of type-V/XI collagen. Type-II collagen was present partly in a pro-α1(II) form, suggesting that there is active synthesis of type-II collagen into the matrix of adult bovine vitreous. Type-IX collagen was purified (2–2.5 mg/l of vitreous) and its glycosaminoglycan chain composition was analysed. Bovine vitreous type-IX collagen always possessed a glycosaminoglycan chain of comparatively low $M_r$ that was predominantly 4-sulphated, with chondroitin 6-sulphate representing a more minor component. By contrast, chick vitreous has been shown to contain type-IX collagen which always possesses a high-$M_r$ chondroitin sulphate chain that is predominantly 6-sulphated. The functional significance of these different glycosaminoglycan chain lengths and sulphation patterns is discussed.

## INTRODUCTION

The vitreous body has previously been shown to contain collagen types II, V/XI and IX by characterization of their pepsinized forms (Ayad and Weiss 1984; Eyre and Wu, 1987; Seery and Davison, 1991). More recently intact type-IX collagen has been extracted from chick and bovine vitreous (Yada et al., 1990; Bishop et al., 1992). Cartilage has similarly been shown to contain collagen types II, IX and XI, and also collagen types VI, XII and XIV (Ayad et al., 1989; Watt et al., 1992).

Collagen types II and V/XI are fibril-forming collagens that in other tissues have been shown to participate in heterotypic (mixed) fibrils (Birk et al., 1988; Mendler et al., 1989). Type-II collagen is a homotrimer $[\alpha1(II)]_3$ and is the predominant collagen type in vitreous (Ayad and Weiss, 1984). Controversy has existed as to whether the other known fibril-forming collagen of vitreous is collagen type V or XI (Ayad and Weiss, 1984; Eyre and Wu, 1987; Seery and Davison, 1991). However, it has been shown recently that bovine vitreous contains collagen monomers assembled from α1 (XI) and α2(V) chains (Mayne et al., 1993). Therefore, in this paper the vitreous collagen will be referred to as collagen type V/XI.

Type-IX collagen has been shown to decorate the surface of the major (heterotypic) collagen fibrils of vitreous in a D-periodic distribution (Ren et al., 1991). It is a heterotrimer [α1(IX)α2(IX)α3(IX)] and is a member of the FACIT (fibril-associated collagens with interrupted triple helices) group of collagens, which also includes collagen types XII, XIV and XVI (van der Rest et al., 1990; Pan et al., 1992). Type-IX collagen possess three collagenous domains COL1, COL2 and COL3 interspersed between four non-collagenous domains NC1, NC2, NC3 and NC4. The N-terminal NC4 domain is short in vitreous (Yada et al., 1990; Brewton et al., 1991; Bishop et al., 1992), probably comprising three amino acids in the α2(IX) and α3(IX) chains and two amino acids in the α1(IX) chain (Muragaki et al., 1990; Ninomiya et al., 1990; Brewton et al., 1992; Har-El et al., 1992). By contrast, the α1(IX) chain of (human) cartilage has a 245 amino acid NC4 domain which forms a globular N-terminal structure that is visible by rotary-shadowing electron microscopy (Vaughan et al., 1988; Muragaki et al., 1990). These different forms of the α1(IX) chain are due to alternative transcription start sites and splicing of the α1(IX) gene (Muragaki et al., 1990). The type-IX collagen of cartilage exists in proteoglycan and non-proteoglycan forms, the proteoglycan form possessing a chondroitin sulphate glycosaminoglycan side chain attached to the α2(IX) component at the NC3 domain (Irwin and Mayne, 1986; Ayad et al., 1991; Yada et al., 1992). By contrast, extractable vitreous type-IX collagen is always in a proteoglycan form, but the length of the glycosaminoglycan chain is species-dependent. The bovine vitreous form was found to possess a comparatively short glycosaminoglycan chain (resulting in type-IX collagen of low buoyant density), whereas the chick vitreous form has a glycosaminoglycan chain of $M_r$ of approx. 350000 (resulting in type-IX collagen of higher buoyant density) (Yada et al., 1990; Bishop et al., 1992).

Previous studies of vitreous collagens have relied upon extracting whole vitreous or centrifuging vitreous and then extracting collagens from the insoluble collagenous residue. We now report that the hyaluronan-rich supernatant obtained following the centrifugation of adult bovine vitreous contains a significant proportion of the total vitreous collagen, and describe the isolation and characterization of these collagens.

## MATERIALS AND METHODS

### Tissue preparation and extraction

Bovine eyes were obtained from a local abattoir and were dissected by making a 360° coronal section behind the vitreous

Abbreviations used: Δdi-HA, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; Δdi-0S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; Δdi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-sulpho-D-galactose; Δdi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-sulpho-D-galactose; Δdi-UA 2S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose.

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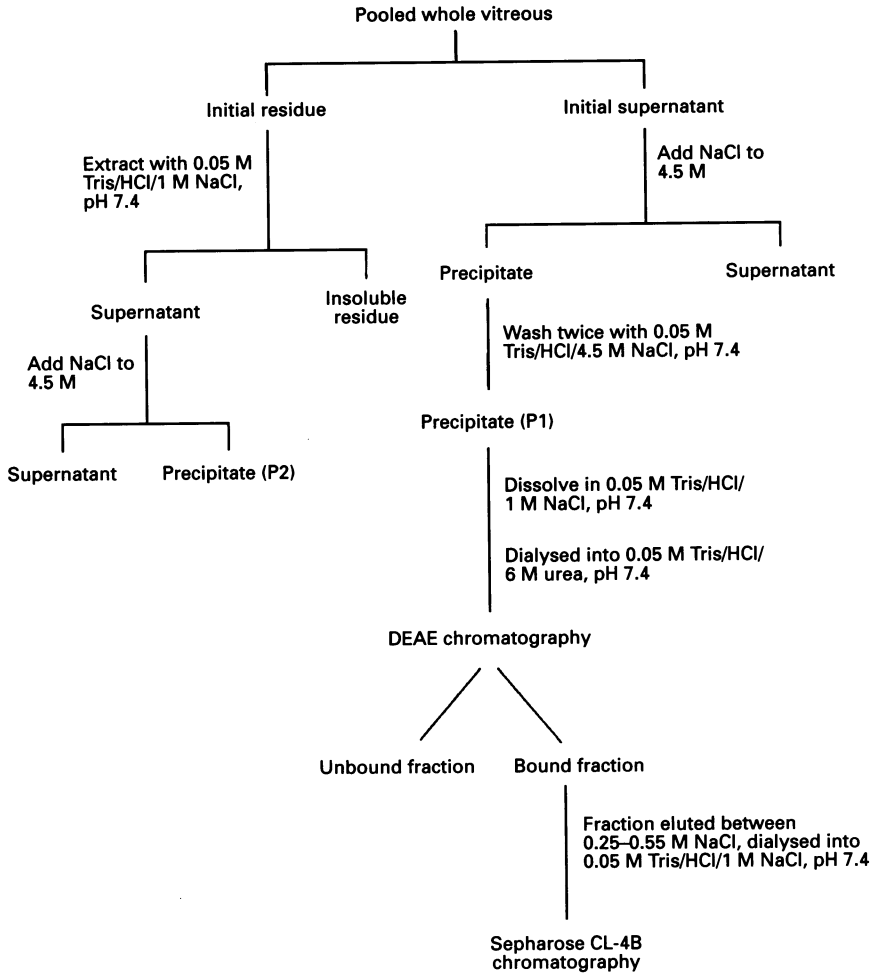


**Figure 1 Flow diagram of the extraction and chromatographic protocols**

base and scooping out the vitreous from the posterior cup. The vitreous was frozen at −30 °C within 3 h of death. On thawing, proteinase inhibitors were added to the pooled vitreous at final concentrations of 2 mM EDTA, 10 mM *N*-ethylmaleimide, 2 mM phenylmethanesulphonyl fluoride and 5 mM benzamidine (unless stated otherwise all reagents were of analytical grade and obtained from Sigma). The vitreous was centrifuged at 30000 *g* for 2 h at 4 °C and then separated into two fractions; the initial supernatant and initial residue (Figure 1). NaCl was added to the initial supernatant to a final concentration of 4.5 M and after stirring overnight at 4 °C the resultant precipitate was pelleted by centrifugation (this and all subsequent centrifugation was carried out at 30000 *g* for 90 min and all procedures were carried out at 4 °C unless otherwise stated).

### Fractionation of initial supernatant

The 4.5 NaCl precipitate from the initial supernatant was washed with 50 mM Tris/HCl/4.5 M NaCl, pH 7.4, (including proteinase inhibitors as above) and centrifuged, twice. The resultant pellet (P1) was redissolved in 50 mM Tris/HCl/1 M NaCl, pH 7.4, (including proteinase inhibitors as above) and clarified. In some experiments aliquots of this fraction were dialysed extensively into 50 mM Tris/HCl/0.15 M NaCl, pH 7.4, and the precipitated material collected by centrifugation.

### DEAE chromatography

P1 (in 50 mM Tris/HCl/1 M NaCl, pH 7.4) was dialysed into 50 mM Tris/HCl, pH 8.3, containing 6 M urea, clarified and applied to a DE52 cellulose (Whatman) column (1.25 cm × 20 cm) equilibrated in the same buffer. The unbound fraction was eluted with the same buffer and the bound fraction eluted with a linear gradient of 0–0.6 M NaCl in this buffer at room temperature. Absorbance was monitored at 230 nm.

### Sepharose CL-4B chromatography

The bound fraction from the DE52 column that was eluted between 0.25 and 0.55 M NaCl in 6 M urea/0.05 M Tris/HCl, pH 8.3, was concentrated by ultrafiltration using an Amicom membrane YM 100 ($M_r$ cut off 100000). The concentrate was dialysed into 50 mM Tris/HCl/1 M NaCl, pH 7.4, and applied to a Sepharose CL-4B column (2 cm × 84 cm) equilibrated in the same buffer. The column was eluted with this buffer at 20 ml/h at room temperature. Absorbance was monitored at 230 nm and fractions pooled as appropriate.

### Extraction of initial residue

The initial residue (Figure 1) was extracted as previously described (Bishop et al., 1992). Briefly, the residue was extracted

twice for 24 h with 50 mM Tris/HCl/1 M NaCl, pH 7.4, containing proteinase inhibitors as above. After centrifugation the two extracts were combined and NaCl added to a final concentration of 4.5 M and the precipitate was pelleted (P2) by centrifugation. The residual insoluble residue (Figure 1) was washed extensively with Milli-Q water and lyophilized.

### Hydroxyproline analyses

Measurements were made using the method of Woessner (1961) modified by replacing 2-methoxyethanol with 1-methylethanol and using 10% (w/v) *p*-dimethylaminobenzaldehyde solution in 1-methylethanol (Ayad and Weiss, 1984). For hydroxyproline analyses of P1, P2 and the insoluble residue, 250 ml samples of pooled whole vitreous were fractionated as described (Figure 1). All of the insoluble residue and fractions P1 and P2 (after redissolving in 0.5 M acetic acid, clarifying, dialysed into 0.1 M acetic acid) were lyophilized and then hydrolysed with 6 M HCl at 105 °C overnight. Aliquots from these hydrolysed fractions were then used for hydroxyproline measurement.

### SDS/PAGE

Samples were analysed in the presence (reduced) and in the absence (non-reduced) of 50 mM dithiothreitol by SDS/PAGE (Laemmli, 1970). The resultant gels were stained with either Coomassie Brilliant Blue R or with silver nitrate by the method of Sammons et al. (1981). Alternatively gels were subjected to Western blotting. Some Coomassie Blue-stained gels were scanned using an LKB 2202 Ultroscan laser densitometer. Some lyophilized samples were treated with chondroitin ABC lyase before electrophoresis by incubating in 100 $\mu$l of 0.05 M Tris/HCl/0.03 M sodium acetate/2 mM phenylmethanesulphonyl fluoride/2 mM EDTA/5 mM benzamidine/10 mM *N*-ethylmaleimide, pH 8, containing 0.01 unit of enzyme/mg of sample for 16 h at 30 °C. Other samples were treated with chondroitin AC-II lyase (Seikagaku) under the same conditions, except that the pH of the buffer was altered to pH 6.

### Western blotting and immunodetection

The production of polyclonal antisera against pepsinized cartilage collagens has been described previously (Ayad et al., 1989): antisera to type-II collagen [anti-(II)] and to the COL1 domain of type-IX collagen [anti-(IX)] were used for immunoblotting. Several antisera which recognize pepsinized collagen type V/XI were used for immunoblotting along with an antiserum which recognizes intact type-V collagen (a generous gift from Dr. Daniel Hartmann, Institut Pasteur de Lyon, France). Transfer to nitrocellulose was by the method of Towbin et al. (1979) with a modified buffer containing 48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS. After transfer, the nitrocellulose sheet was immunoblotted with the primary antiserum (1:1000 dilution) followed by the secondary alkaline-phosphatase-conjugated antiserum (1:1000) by the method of Blake et al. (1984).

Some Western blots were performed on Immobilon P membrane (Millipore) and incubated with a polyclonal antiserum which recognizes bovine decorin and biglycan (Sampaio et al., 1988) or an anti-decorin monoclonal antibody 6D6 (Pringle et al., 1985); both were used at a dilution of 1:1000 in 1% (w/v) Marvel/PBS. After incubation with peroxidase-conjugated secondary antibody [diluted 1:1000 in 1% (w/v) Marvel/PBS] detection was performed using the enhanced chemiluminescence Western blotting analysis system (Amersham International) according to manufacturer's instructions. In these cases bovine skin decorin, bovine articular cartilage decorin and biglycan and pig articular cartilage decorin and biglycan (all generously provided by Dr. Gill Venn, Kennedy Institute of Rheumatology, London) were used as positive controls.

### Pepsinization of samples

Samples were suspended in 0.5 M acetic acid (pH 2.5) and incubated with pepsin (1:50 enzyme:substrate ratio) for 24 h. In some experiments pepsin-solubilized material was fractionated by differential salt precipitation at 0.86 M, 1.2 M and 3 M NaCl.

### Analyses of sulphated glycosaminoglycan chains

The glycosaminoglycan composition of purified type-IX collagen (from the initial supernatant) and the lyophilized insoluble residue (Figure 1) were analysed by capillary zone electrophoresis (Carney and Osborne, 1991). Samples were digested with chondroitin ABC lyase in 0.1 M Tris/HCl, pH 7.9, for 3 h at 37 °C. Purified type-IX collagen (150 $\mu$g) was suspended in 25 $\mu$l of buffer and digested with 0.05 unit of chondroitin ABC lyase; insoluble residue (10 mg) was suspended in 200 $\mu$l of buffer and digested with 0.25 unit of chondroitin ABC lyase. After centrifugation at 10000 *g* for 10 min the supernatant was collected and lyophilized. The lyophilized supernatant was redissolved in 40 mM $Na_2HPO_4$, 40 mM SDS, 10 mM sodium tetraborate, pH 9.0, and subjected to capillary zone electrophoresis at 15 kV, 40 °C (Model 270A, Applied Biosystems, Cheshire, U.K. interfaced with a Drew DS4000 integration package). Resultant peak integrals were compared with those for standard peak integrals obtained from unsaturated monosulphated and unsulphated disaccharides (Seikagaku, Tokyo).

The proportion of the total glycosaminoglycan released by chondroitin ABC lyase digestion of the insoluble residue was analysed. After chondroitin ABC lyase digestion and centrifugation (as above) the insoluble residue was digested with 0.013% (w/v) papain (in 0.5 M Tris/HCl/20 mM EDTA/20 mM cysteine hydrochloride, pH 6.8) for 16 h at 60 °C. The uronic acid content of the supernatant and the papain-digested insoluble residue was determined by the method of Bitter and Muir (1962).

## RESULTS

The total hydroxyproline content of wet vitreous (estimated by addition of values for the fractions P1, P2 and the insoluble residue, Figure 1) was 9.5 $\mu$g/g. Fractionation of the whole vitreous by centrifugation resulted in the formation of an initial residue and initial supernatant (Figure 1). Approx. 96% of the hydroxyproline was associated with the initial residue and less than 1% of this could be extracted with 1 M NaCl (Figure 1, P2). The initial supernatant contained on average 4.2% of the total collagen and this collagen could be precipitated from the initial supernatant by the addition of 4.5 M NaCl (Figure 1, P1). This precipitate was redissolved and used to isolate and characterize intact vitreous collagens.

Figure 2 shows a representative SDS/PAGE analysis of the P1 fraction (Figure 1) after chondroitin ABC lyase digestion. Laser densitometric scanning indicated that type-IX collagen was the predominant collagen type, with collagen types IX and II occurring in the approximate ratio of 4:1. By contrast, when the

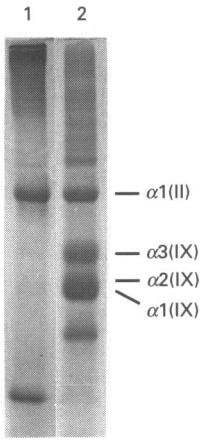


**Figure 2 SDS/PAGE (6.5% acrylamide gel) of the 4.5 M NaCl precipitate (P1) of the initial supernatant fraction (Figure 1) after chondroitin ABC lyase digestion**

Lane 1 was unreduced, whereas lane 2 was reduced. The α1(IX) component and α2(IX) component after chondroitin ABC lyase digestion nearly always co-migrated.

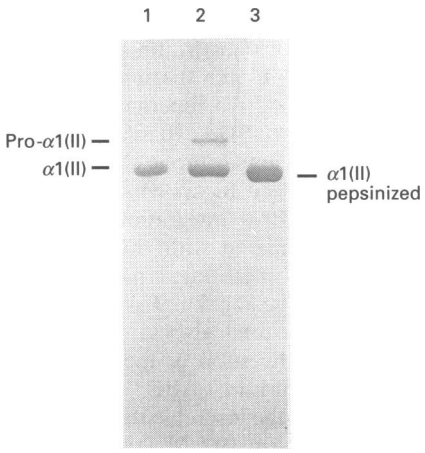


**Figure 3 Immunoblot of the 4.5 M NaCl precipitate (P1) from the initial supernatant fraction (Figure 1) with anti-(II)**

Equal aliquots were analysed by SDS/PAGE (7.5% acrylamide gel) before and after reduction and immunoblotted with anti-(II) [an anti-(type-II collagen) serum]. Lane 1 was unreduced and not pepsinized; lane 2 was reduced and not pepsinized; lane 3, the sample was pepsinized before SDS/PAGE and immunoblotting.

insoluble residue (Figure 1) was pepsinized, salt-fractionated and analysed by gel scanning and hydroxyproline determinations the ratio of collagens II:V/XI:IX was found to be 15:2:3 (results not shown).

Immunoblotting of the P1 fraction (Figure 1) with anti-(II) revealed the intact α1(II) component (Figure 3, lane 1). The vitreous α1(II) component migrated slightly above cartilage α1(II) on SDS/PAGE on 6.5% polyacrylamide gels (results not shown). Upon reduction a further prominent component was immunoblotted with anti-(II) which migrated more slowly than α1(II) (Figure 3, lane 2). Pepsinization of the P1 fraction resulted in a single component that migrated slightly faster than intact α1(II) on SDS/PAGE (Figure 3, lane 3). These results indicate that the component which migrated more slowly than α1(II) and immunoblotted with anti-(II) was pro-α1(II). After DE52 chromatography the intact (fully processed) α1(II) component was eluted in the unbound fraction and appeared, by SDS/PAGE, to be the only constituent of this fraction (Figure 4b, lane 1),whereas pro-α1(II) was eluted in the bound fraction between 0 and 0.25 M NaCl (lane 2).

Type-II collagen could be easily identified in P1 (Figure 1), but type-V/XI collagen could not be identified by immunoblotting with an antiserum directed against the intact collagen. Attempts were made to selectively precipitate type-V/XI collagen by dialysis into a low-ionic-strength buffer (0.05 M Tris/HCl/0.15 M NaCl, pH 7.4) and immunoblotting the resultant precipitate, but intact type-V/XI collagen could not be detected. The P1 and P2 precipitates (Figure 1) were pepsinized and fractionated by differential salt precipitation. Immunoblots of these fractions with antisera which react strongly to pepsinized collagen V/XI from the insoluble residue (results not shown) demonstrated that type-V/XI collagen was a very minor component of precipitate P1 (producing very faint bands, results not shown) and could not be demonstrated at all in fraction P2.

While type-II collagen is the major collagen type in unfractionated vitreous and the insoluble residue (Figure 1), the predominant collagen type in the initial supernatant (Figure 1) was type-IX collagen (Figure 2). Type-IX collagen was isolated from other vitreous proteins by a combination of DE52 ion-exchange chromatography and Sepharose CL-4B gel-filtration chromatography. Following DE52 ion-exchange chromatography all of the type-IX collagen was eluted in the bound fraction (Figure 4b). Immunoblotting of bound DE52 fractions with anti-(IX) (Figure 4c) showed the glycanated α2(IX) component [i.e. the α2(IX) chains with attached glycosaminoglycan] as diffuse bands with a gradated increase in size as the NaCl concentration was increased up to 0.55 M. The fractions that were eluted from the DE52 cellulose column between 0.25 and 0.55 M NaCl were pooled, as these contained most of the type-IX collagen (Figure 4b). The pooled fraction was concentrated and applied to a Sepharose CL-4B column (Figure 5). SDS/PAGE (under reducing conditions) of the pooled fraction that was eluted between 50 and 70 ml from the Sepharose CL-4B column ($V_0$ 64 ml) failed to show any visible components after Coomassie Blue staining, and the pooled fractions between 98 and 180 ml contained predominantly albumin (results not shown). The fractions which were eluted between 71 and 97 ml (indicated by the arrowed bar in Figure 5a) were collected and analysed by SDS/PAGE and immunoblotting with anti-(IX) (Figure 5). Figure 5(b) shows the SDS/PAGE and Coomassie Blue staining of the pooled fractions which were eluted between 71 and 97 ml on Sepharose CL-4B chromatography. The unreduced sample produced a diffuse band at the top of the gel (Figure 5b, lane 1), which after pretreatment with chondroitin ABC lyase migrated slightly further into the gel (lane 2). Upon reduction (Figure 5b, lane 3) the α1(IX) and α3(IX) bands were clearly visible, but the diffuse band produced by the glycanated α2(IX) component was not clearly discernible by Coomassie Blue staining. After pretreatment with chondroitin ABC lyase or chondroitin AC-II lyase (Figure 5b, lanes 4 and 5) the α2(IX) component co-migrated with the α1(IX) component, producing a single band of increased intensity as compared with α1(IX) alone (lane 3). When the pooled fractions which were eluted between 71 and 97 ml on Sepharose CL-4B were immunoblotted with anti-(IX) the glycanated α2(IX) component was clearly discernible as a diffuse band (Figure 5c, lane 1). After pretreatment with chondroitin ABC lyase the diffuse band produced by the α2(IX) was removed and the α1(IX) and α2(IX)

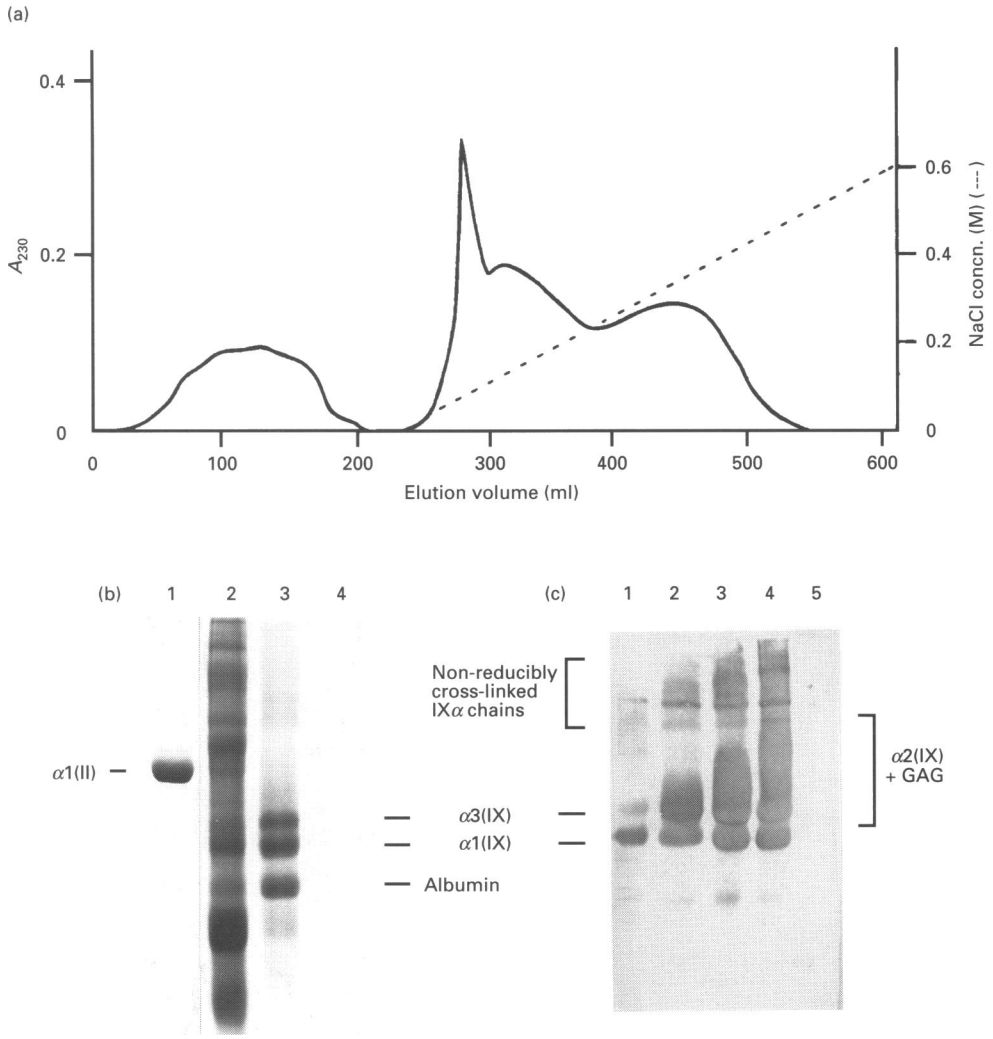


**Figure 4 Chromatography of fraction P1 (Figure 1) on a DEAE-cellulose ion-exchange column and SDS/PAGE and anti-(IX) immunoblot of resultant fractions**

The P1 fraction was redissolved in 50 mM Tris/HCl/1 M NaCl, pH 7.4, and then equilibrated in 6 M urea/50 mM Tris/HCl, pH 8.3. The solubilized material was applied to a DE52-cellulose ion-exchange column (1.25 cm × 20 cm) and was eluted with the same buffer at a rate of 30 ml/h until the non-retarded material was eluted. A linear NaCl gradient of 0–0.6 M NaCl was then applied over a volume of 350 ml. (**a**) A representative elution profile. (**b**) Coomassie Blue-stained gel after reduction and SDS/PAGE (6.5% acrylamide gel) of unbound (lane 1) and bound fractions eluted at 0–0.25 M NaCl (lane 2); 0.25–0.55 M NaCl (lane 3); 0.55–0.6 M NaCl (lane 4). (**c**) Immunoblot with anti-(IX) of bound fractions were eluted from DE52 with 0–0.6 M NaCl. Samples were reduced and subjected to SDS/PAGE (6.5% acrylamide gel) before immunoblotting. Lane 1, 0–0.15 M NaCl; lane 2, 0.15–0.2 M NaCl; lane 3, 0.2–0.25 M NaCl; lane 4, 0.25–0.55 M NaCl; lane 5, 0.55–0.5 M NaCl.

chains co-migrated (Figure 5c, lane 2). Similarly chondroitin AC-II lyase completely removed the diffuse band immunoblotted with anti-(IX) (Figure 5c, lane 3), suggesting that the glycosaminoglycan chain attached to α2(IX) is predominantly chondroitin as opposed to dermatan sulphate.

SDS/PAGE under reducing conditions and Coomassie Blue staining of the pooled fractions which were eluted between 71 and 97 ml from the Sepharose CL-4B column showed non-reducible high-relative-molecular-mass components with $M_r$ values of approx. 2- and 3-fold that of single type-IX collagen α-chains (Figure 5b, lanes 3–5). These non-reducible components were immunoreactive with anti-(IX) (Figures 4c and 5c, lanes 2 and 3), but not with anti-(II) (results not shown), suggesting that they represented type-IX collagen α-chains linked by lysine/hydroxylysine-derived aldehyde cross-links. In addition to the group of three bands which showed an electrophoretic mobility compatible with two α(IX) chains linked by non-reducible bonds, a fourth component was observed after digestion with chondroitin ABC lyase or chondroitin AC-II lyase (Figure 5b, asterisk in lanes 4 and 5). When the fractions which bound to the DE52-cellulose column were immunoblotted with anti-(IX) (Figure 4c) diffuse components were visible above the main non-reducibly cross-linked dimers of α(IX) chains, which had a gradated increase in size with increasing salt concentration. Furthermore, after glycosidase digestion (Figure 5b, lanes 4 and 5) components were visible that just migrated into the gel in a position similar to that shown by chondroitin ABC lyase-pretreated, unreduced type-IX collagen (Figure 5b, lane 2). All these results suggest that the non-reducibly cross-linked type-IX collagen α-chains sometimes included glycanated α2(IX) chain(s).

Analysis of the fraction which was eluted from the Sepharose CL-4B column between 71 and 97 ml by SDS/PAGE with Coomassie Blue staining and immunoblotting (Figures 5b and 5c) suggested that type-IX collagen was the only component in

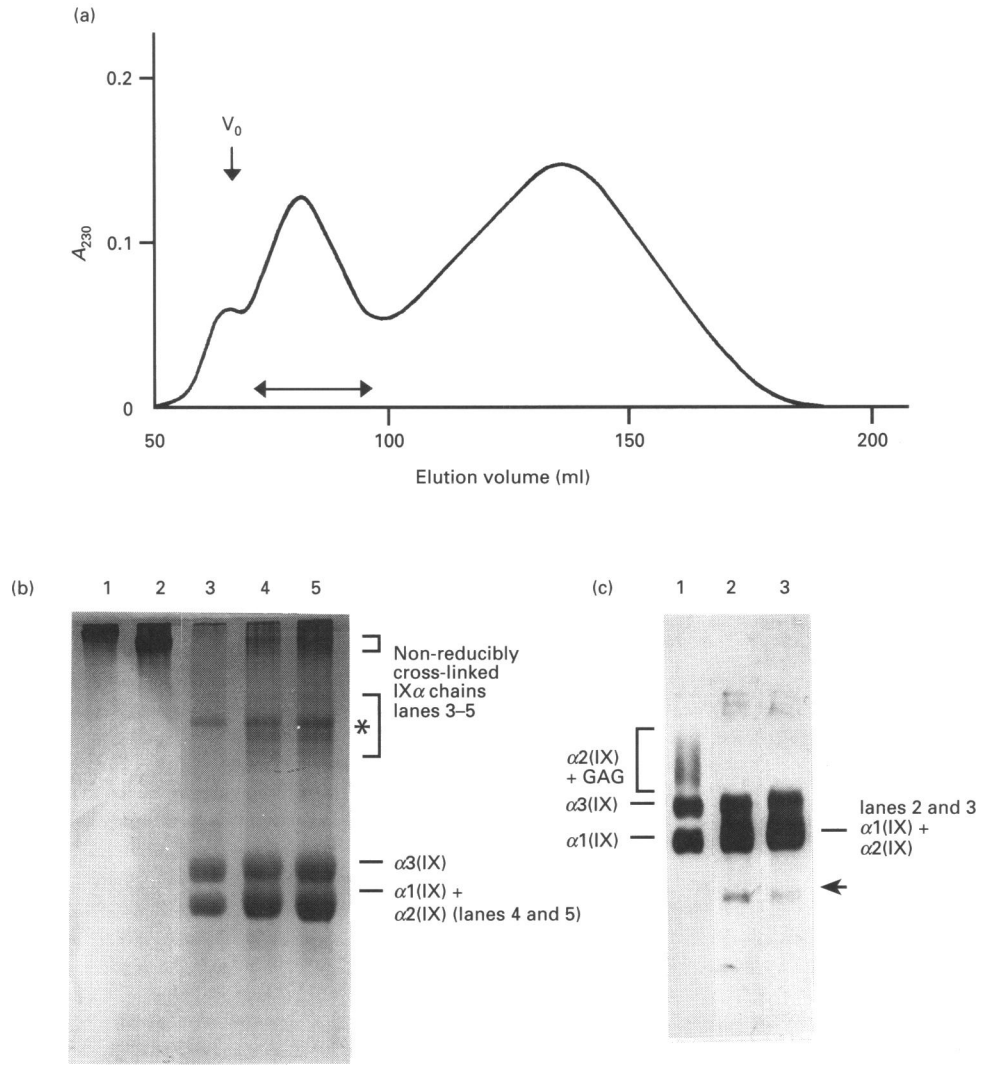


**Figure 5 Sepharose CL-4B molecular-sieve chromatography of type-IX collagen**

The fractions which were eluted from DEAE-chromatography between 0.25 and 0.55 M NaCl were pooled, concentrated by ultrafiltration, equilibrated in 50 mM Tris/HCl/1 M NaCl, pH 7.4, applied to a Sepharose CL-4B column (2 cm × 84 cm) and eluted with the same buffer at 20 ml/h. Fractions delineated by the arrowed bar (between 71 and 97 ml) were pooled and analysed by SDS/PAGE and immunoblotting with anti-(IX). (**a**) Representative elution profile. (**b**) SDS/PAGE (6.5% acrylamide gel) of pooled fractions which were eluted between 71 and 97 ml and stained with Coomassie Blue. Lane 1, unreduced; lane 2, unreduced plus chondroitin ABC lyase; lane 3, reduced; lane 4, reduced plus chondroitin ABC lyase; lane 5, reduced plus chondroitin ACII lyase. A number of non-reducible cross-links between α(IX) chains were present including one (indicated by *) which appears after glycosidase digestion. (**c**) Immunoblot with anti-(IX) of pooled fractions which were eluted between 71 and 97 ml following reduction and SDS.PAGE (6.5% acrylamide gel). Lane 1, untreated with glycosidic enzyme; lane 2, pretreated with chondroitin ABC lyase; lane 3, pretreated with chondroitin ACII lyase (← indicates albumin present in the glycosidic enzyme preparations).

this fraction. However, further evidence for the purity of this fraction was obtained; in particular to exclude the presence of small proteoglycans which may contribute to the glycosaminoglycan analytical values. The sample was analysed by SDS/PAGE with (lane 1) and without (lane 2) chondroitin ABC lyase pretreatment and the resultant gel stained with silver nitrate (Figure 6). A prominent band (albumin) and a number of minor bands appeared after chondroitin ABC lyase pretreatment, but these exactly matched the pattern of bands produced by chondroitin ABC lyase alone (lane 3). The enzyme/substrate ratio of chondroitin ABC lyase to the type-IX-collagen-containing fraction was 3:100 (w/w). Consequently, there was far less than 3% contamination of the fraction by silver nitrate-stained impurities, as all the additional visible components following chondroitin ABC lyase digestion were attributable to the enzyme itself.

Proteoglycans may, however, stain poorly with silver stains even after enzymic removal of the glycosaminoglycan side chains. Therefore, the P1 fraction and the fraction which was eluted from the Sepharose CL-4B column between 71 and 97 ml (purified type-IX collagen) were analysed by SDS/PAGE and immunoblotting for the small proteoglycans decorin and biglycan (Figure 7). While the positive controls (lanes 3–6) were clearly visible on the immunoblots (decorin $M_r$ approx. 100000 and biglycan $M_r$ approx. 200000), no antibody binding was visible in the lanes containing the P1 fraction (lane 2) or the purified type-IX collagen (lane 1). Therefore, these results suggested that the purified type-IX collagen fraction was not contaminated by small proteoglycans and that analyses of its glycosaminoglycan composition would reflect that of type-IX collagen in bovine vitreous. Intact purified type-IX collagen was prepared (2.0–2.5 mg/l of vitreous) and consequently ample amounts of

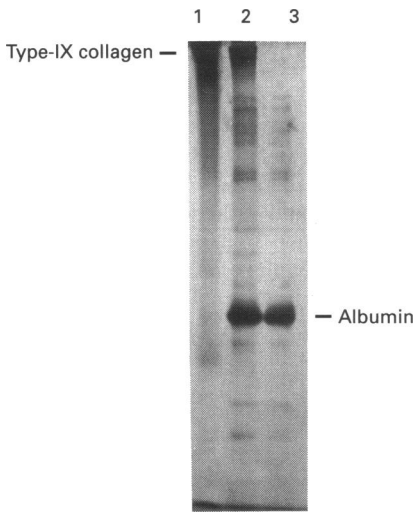


**Figure 6 SDS/PAGE of 'purified type-IX collagen'**

Fractions that were eluted from the Sepharose CL-4B column between 71 and 97 ml were pooled and analysed by SDS/PAGE (8% polyacrylamide gel) under non-reducing conditions, without (lane 1) and with (lane 2) chondroitin ABC lyase pretreatment. Lane 3 contains chondroitin ABC lyase alone. The gel was stained with silver nitrate.

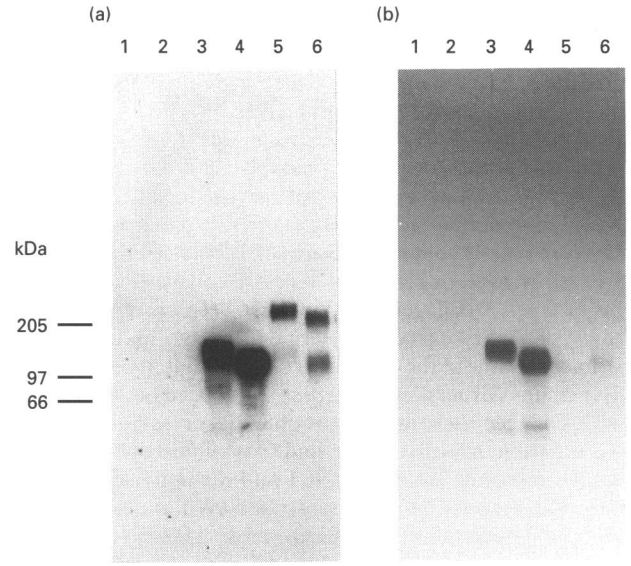


**Figure 7 Immunodetection of decorin and biglycan**

Fractions were analysed on a 4–20% linear gradient polyacrylamide gel under reducing conditions and after transfer immunoblotted with specific antisera generated against (**a**) decorin and biglycan, and (**b**) against decorin (6D6). In each case the lanes were as follows: lane 1, purified type-IX collagen from bovine vitreous; lane 2, P1 fraction from bovine vitreous; lane 3, a semi-purified preparation of pig decorin; lane 4, purified bovine skin decorin; lane 5, a semi-purified preparation of pig articular cartilage biglycan; lane 6, a crude guanidinium chloride extract from bovine articular cartilage.

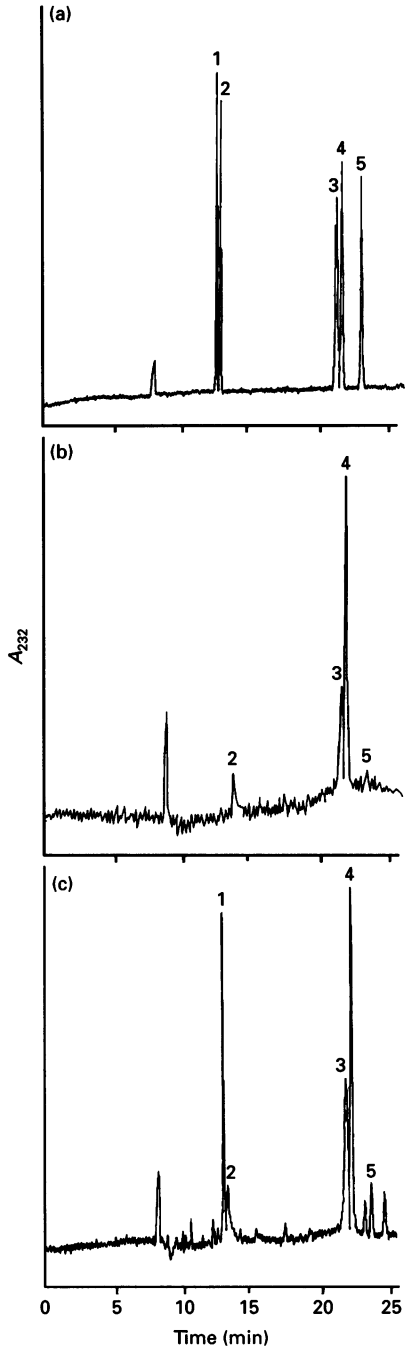


**Figure 8 Electrophoretograms of unsaturated disaccharides produced by chondroitin ABC lyase digestion**

Electrophoresis was carried out at 15 kV, 40 °C in 40 mM phosphate/40 mM SDS/10 mM borate, pH 9.0, on a 72 cm column, 50 $\mu$m internal diameter. (**a**) Standard unsaturated disaccharides. Peak 1 is Δdi-HA, peak 2 is Δdi-0S, peak 3 is Δdi-6S, peak 4 is Δdi-4S, peak 5 is Δdi-UA 2S (see abbreviation footnote on title page). Representative traces for (**b**) purified type-IX collagen and (**c**) the insoluble residue after extracting with 50 mM Tris/HCl/1 M NaCl, pH 7.4, and washing extensively with Milli-Q water.

this proteoglycan were available for analysis of its glycosaminoglycan chain.

The glycosaminoglycan composition of the purified type-IX collagen and that of the insoluble collagenous residue (Figure 1) were analysed by digestion with chondroitin ABC lyase and separation of the resultant disaccharides by capillary zone electrophoresis (Figure 8). Approx. 30% of the total uronic acid in the insoluble residue was released by chondroitin ABC lyase digestion. Repeated analyses were performed on the purified type-IX collagen from three separate preparations and on the

**Table 1 Composition of glycosaminoglycans obtained from purified type-IX collagen and from the insoluble collagenous residue (Figure 1)**

Shown are mean percentage values for the release of unsulphated and monosulphated unsaturated disaccharides by digestion with chondroitin ABC lyase. Figures in parentheses are S.D.s.

| Disaccharide units | Purified type-IX collagen | Insoluble residue |
|---|---|---|
| Δdi-0S | 18 (9) | 10 (7) |
| Δdi-4S | 49 (7) | 48 (4) |
| Δdi-6S | 28 (6) | 34 (4) |
| Δdi-UA 2S | 5 (3) | 8 (5) |

disaccharides released from the insoluble residues from four separate experiments. Mean values were obtained from these analyses and these are shown as percentages of released monosulphated and unsulphated disaccharides from chondroitin/dermatan sulphate (Table 1). The results show that the glycosaminoglycans released from the purified type-IX collagen and those of the insoluble residue were very similar, both being predominantly 4-sulphated (Δdi-4S) (approx. 50%), with chondroitin 6-sulphate (Δdi-6S) representing a more minor component (approx. 30%). Additionally, there were lesser amounts of unsulphated chondroitin (Δdi-0S) and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose (Δdi-UA 2S).

## DISCUSSION

Vitreous collagens have been isolated previously by extracting whole vitreous or centrifuging the vitreous and extracting the insoluble residue. We report that after centrifugation of adult bovine vitreous, the hyaluronan-rich supernatant (initial supernatant) contains approx. 4% of the total vitreous collagen and that collagen types IX and II are present in the ratio 4:1, with negligible amounts of type-V/XI collagen. By contrast, we found that the insoluble residue contained collagens II, V/XI and IX in the ratio of 15:2:3, which is in agreement with our previous analyses of 77:8:15 (Ayad and Weiss, 1984) and those of Ren et al. (1991) who also found that 15% of the collagen is type IX. A different ratio (69:24:7) was reported by Seery and Davison (1991). However, all these results show that a larger proportion of type-II collagen was present relative to type-IX collagen in the collagenous residue. The significance of the predominance of type-IX collagen in the initial supernatant is unclear though it is possible that the type-IX collagen is involved in the formation of distinct supramolecular assemblies other than those within the main heterotypic collagen fibrils.

Collagen types I and V, and II, IX and XI have been shown to form heterotypic (mixed) fibrils in cornea and cartilage respectively. Similarly, in vitreous it is likely that the collagen types II, V/XI and IX form heterotypic fibrils. The relationship between the type-II and type-V/XI collagens in the heterotypic fibrils of vitreous remains unknown, but in other tissues it has been shown that the triple-helical components of collagen types V/XI are buried within the heterotypic fibrils *in vivo* (Birk et al., 1988; Mendler et al., 1989). The relative lack of type-V/XI collagen in the initial vitreous supernatant fractions, and the finding that the triple-helical components of collagen types V/XI are buried within heterotypic fibrils in other tissues, supports the hypothesis that type-V/XI collagen is relatively insoluble under physiological conditions and may be involved in the initial nucleation of fibrillogenesis (Eikenberry et al., 1992).

We provide the first evidence for the presence of pro-α1(II), and hence active synthesis of this collagen in the adult vitreous. It has been shown that there is an increase in hydroxyproline concentration with age in bovine eyes (Swann and Constable, 1972), suggesting that there is post-natal synthesis of vitreous collagen up to adulthood. Furthermore, although adult cartilage collagen has been shown to contain approx. twice as many mature 3-hydroxypyridinium cross-links as compared with adult vitreous collagen, the vitreous collagen possesses five times the amount of immature dehydro-dihydroxylysinonorleucine cross-links compared with cartilage collagen (Snowden et al., 1982). These authors suggested that this may either be due to incomplete maturation of the 3-hydroxypyridinium cross-links or active collagen synthesis in adult vitreous, which was occurring at a faster rate than that of adult cartilage.

Previously, we investigated the very small amounts of intact type-IX collagen that were extractable from the insoluble residue following centrifugation of bovine vitreous (in essence precipitate P2, see Figure 1) and demonstrated that the three chains of type-IX collagen have approximate $M_r$ values of α1(IX) 64000, α2(IX) after chondroitin ABC lyase digestion 67000, and α3(IX) 78000 (Bishop et al., 1992). Furthermore, we demonstrated that bovine vitreous type-IX collagen always possesses a short glycosaminoglycan chain and has a low buoyant density (< 1.34 g/ml). We now show that much larger quantities of intact type-IX collagen can be extracted from the (initial) supernatant after centrifugation of whole vitreous, allowing a more detailed characterization of this molecule; in particular we have identified non-disulphide-bonded cross-links between α(IX) chains (which are probably lysine/hydroxylysine-derived aldehyde cross-links) and we have been able to purify type-IX collagen and analyse its glycosaminoglycan composition.

The lack of immunoreactivity of the irreducibly cross-linked α(IX) chains to anti-(II) suggests that these cross-linked forms do not involve type-II collagen and are solely between the two, three and possibly more α-chains of type-IX collagen. Cross-linked forms of type-IX collagen have similarly been observed in organ cultures of embryonic chick sternal cartilage and, as in our experiments, some of the cross-linked forms contained glycanated α2(IX) chains (Bruckner et al., 1985). All three bovine cartilage type-IX collagen α-chains possess potential cross-linking sites in the COL2 triple-helical domain, and cross-links between type-IX molecules involving the α3(IX)NC1 and the major COL2 cross-linking domain have been demonstrated (Wu et al., 1992). Our results could not confirm whether the cross-links in vitreous were intramolecular or intermolecular, but the presence of four components with an $M_r$ value consistent with two cross-linked α(IX) chains suggests that intermolecular bonds may be present, as there are only three possible combination pairs of α(IX) chains involving intramolecular bonds. Further studies of these cross-linked forms are currently being undertaken.

Several criteria suggested that a highly purified sample of intact type-IX collagen was obtained from the initial supernatant including (1) failure to detect other components on SDS/PAGE (including a highly sensitive silver staining technique, Figure 6); (2) the exclusion by gel-filtration chromatography of high- and low-$M_r$ components; (3) the fractions collected after gel-filtration chromatography were derived from a single peak by absorbance at 230 nm; and (4) the presence of the small proteoglycans decorin and biglycan were excluded specifically by immunoblotting.

The analyses of the glycosaminoglycan chain of the purified type-IX collagen are shown in Table 1. As a large proportion of

the type-IX collagen in vitreous is cross-linked to the major heterotypic collagen fibrils and remains in the insoluble residue (Figure 1), the disaccharides released by chondroitin ABC lyase digestion of this fraction were analysed (Table 1). Uronic acid analyses demonstrated that 30% of the glycosaminoglycan in the insoluble residue was released by chondroitin ABC lyase digestion and therefore the disaccharide analyses may have been unrepresentative due to the preferential release of certain types of disaccharide by this enzyme. However, the proportions of monosulphated disaccharides released from the insoluble residue were very similar to those released from the purified type-IX collagen, suggesting that the glycosaminoglycans associated with the insoluble residue were derived from type-IX collagen and that at least a portion of the type-IX collagen that was covalently linked to the major collagen fibrils was glycanated. Electron microscopic studies using Cupromeronic Blue with critical electrolyte techniques demonstrated sulphated glycosaminoglycans extending away from the major collagen fibrils of vitreous in a D-periodic distribution, occasionally bridging adjacent fibrils (Scott, 1992), and type-IX collagen has similarly been shown to be D-periodically distributed along the surface of the major collagen fibrils of mammalian vitreous by rotary-shadowing electron microscopy (Ren et al., 1991). It is likely that these two techniques are observing different components of the same type-IX collagen molecules.

The comparatively short glycosaminoglycan chains of bovine vitreous type-IX collagen are predominantly 4-sulphated, whereas the type-IX collagen of fetal chick vitreous possesses very large (approx. 350000-$M_r$) chondroitin 6-sulphate chains (Yada et al., 1990). The major glycosaminoglycan of chick vitreous gel is chondroitin sulphate which is thought to be contributed by the long glycosaminoglycan chains of type-IX collagen. In contrast, the predominant glycosaminoglycan of mammalian vitreous in hyaluronan and sulphated glycosaminoglycans represent a minor component (Allen et al., 1977). Both hyaluronan and chondroitin 6-sulphate are capable of self-associating and are able to form the extended filamentous glycosaminoglycan networks which are found in vitreous, whereas chondroitin 4-sulphate is not capable of forming such networks (Scott et al., 1991, 1992; Brewton and Mayne, 1992). Chondroitin 4-sulphate may, however, be able to form heteroduplexes with hyaluronan (Scott et al., 1992), and chondroitin 6-sulphate has been shown to interact specifically with hyaluronan *in vitro* (Turley and Roth, 1980). Therefore, the relatively short glycosaminoglycan chains of bovine vitreous type-IX collagen are not needed to form an extended filamentous glycosaminoglycan meshwork in the hyaluronan-rich mammalian vitreous. Instead, they may provide a mechanism whereby the major collagen fibrils of bovine vitreous can interact with the hyaluronan meshwork (via chondroitin 4- and 6-sulphate) or with adjacent collagen fibrils (via chondroitin 6-sulphate duplexes) and consequently may be of key importance in the supramolecular assembly of bovine vitreous.

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