The Isolation of Collagen-Associated Proteoglycan from Bovine Nasal Cartilage and its Preferential Interaction with α_2 Chains of Type I Collagen

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A collagen complex from bovine nasal cartilage was prepared by extraction of the tissue with 3M-MgCl₂ solutions, by using two different procedures. When it was compared with calf skin acid-soluble tropocollagen by polyacrylamide-gel electrophoresis, the 3M-MgCl₂-soluble cartilage collagen in the complex appeared to be predominantly type I in nature, consisting of both α_1 and α_2 chains. The soluble cartilage collagens were digested with purified bacterial collagenase, and the soluble digests were fractionated on Sepharose 4B. Hydroxyproline-free proteoglycan was isolated in the excluded volume of the column eluate, and this was found to be an aggregate which could be dissociated to link proteins and proteoglycan subunit by equilibrium-density-gradient centrifugation in a CsCl–4M-guanidinium chloride gradient. Interaction with calf skin acid-soluble tropocollagen was studied by CM-cellulose chromatography. The link-protein system did not interact, but proteoglycan from the bottom of the gradient did interact. In addition, when proteoglycan subunit was allowed to interact with collagen, there was a preferential binding to the α_2 and β_{12} components, and this effect was also observed with the proteoglycan material obtained from the collagenase digests of 3M-MgCl₂-soluble cartilage collagen complexes. However, specificity for α_2 and β_{12} chains was not exhibited by chondroitin sulphate glycosaminoglycan, and it is therefore concluded that preference for α_2 and β_{12} chains is a function of the intact proteoglycan structure.

The proteoglycan components of bovine nasal cartilage have been extensively studied and their aggregation and dissociation properties elucidated (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969; Gregory, 1973; Hascall & Heinegard, 1974*a,b*; Heinegard & Hascall, 1974). Bovine nasal cartilage collagen, however, has only been identified as CNBr peptides released from 4M-guanidinium chloride-extracted cartilage (Miller & Lunde, 1973). These peptides were characteristic of a type II collagen, with three identical polypeptide chains.

The object of the work described here was to investigate the relationship between collagen and non-collagenous components of bovine nasal cartilage. Collagenase digestion was used to isolate collagen-associated molecules, and interactions between these molecules and collagen were demonstrated *in vitro*. The results were compared with those obtained from similar interaction experiments between cartilage proteoglycan and acid-soluble calf skin tropocollagen. The interaction experiments were based on CM-cellulose chromatography of tropocollagen, and this method enabled the degree of interaction of proteoglycan with individual collagen chains to be assessed.

Materials

All reagents were of analytical grade except for glucuronolactone, carbazole and guanidinium chlor-

ide. Crude *Clostridium histolyticum* collagenase (lot 5041; P-L Biochemicals, Milwaukee, Wis., U.S.A.) was purified until it was virtually proteinase-free (Lee-Own & Anderson, 1975). Chondroitin sulphate was from BDH Chemicals, Poole, Dorset, U.K., and hyaluronic acid was a gift from Dr. M. B. Mathews (University of Chicago, Chicago, Ill., U.S.A.).

Methods

Analytical methods

Hexuronic acid was determined by the method of Bitter & Muir (1962) with glucuronolactone as standard. Hydroxyproline was determined by the method of Woessner (1961) after hydrolysis in 6M-HCl at 110°C for 16h. Acid was removed by rotary evaporation and the sample was dissolved in a known volume of water for analysis. Monosaccharides were determined by g.l.c., by the method of Clamp *et al.* (1971). A Pye series 104 gas chromatograph was used, with a temperature programme from 120° to 200°C at 1.5°C/min.

Analytical polyacrylamide-gel electrophoresis was performed on 5% (w/v) polyacrylamide in the presence of sodium dodecyl sulphate and urea by the method of Furthmayr & Timpl (1971). This method allowed the identification of the individual α and β components of collagen, and was also utilized for the

estimation of molecular weights based on the linear relationship between mobility of proteins and their molecular weight. Standards used were bovine serum albumin monomer and dimer [from bovine serum albumin fraction V; Sigma (London) Chemical Co., London S.W.6, U.K.] and IgG* (Miles Laboratories, Stoke Poges, Bucks., U.K.). Molecular weights were taken as 68000, 136000 and 150000 respectively.

Preparation of 3M-MgCl₂-soluble collagen complexes

Bovine nasal septa, excised from cattle within 2 h of death, were dissected free of adhering tissue and perichondrium, frozen at -20°C , and shredded with a Stanley Surform (Stanley Tools, Sheffield, U.K.). Two general methods for extracting the complexes were used.

Series A. The method used was essentially that of Anderson & Jackson (1972). Shredded cartilage (wet wt. 332 g) was extracted over a period of 14 days at 4°C with 10×1 litre portions of 0.15M-NaCl – $0.02\text{M-NaH}_2\text{PO}_4$ – 0.01% (w/v) NaN_3 , pH 7.4 (phosphate-buffered saline). The extracts were separated by centrifugation at $1500g$ for 20 min at 4°C , and the supernatant was discarded. The residue was washed finally with water (2×1 litre) to remove traces of phosphate, and was extracted twice with 3M-MgCl_2 . Each extraction was for 3 days at 4°C with 3 litres

* Abbreviation: IgG, immunoglobulin G.

of solution. Soluble extracts were separated by centrifugation ($2000g$ for 30 min, 4°C) and dialysed for 3 days at room temperature. A precipitate resulted from the second extraction and this was separated by centrifugation ($2000g$ for 30 min, 4°C), and extracted twice with portions of phosphate-buffered saline (200 ml) over periods of 24 h. The residual material (3M-MgCl_2 -soluble collagen complex) was dialysed and stored frozen at -20°C until required.

Series B. Shredded cartilage (wet wt. 260 g) was extracted repeatedly with portions (4 litres) of 3M-MgCl_2 over periods of 3 days at 4°C . Soluble extracts were separated and dialysed as for Series A. The first two extracts did not form precipitates on dialysis. The third 3M-MgCl_2 extract yielded a precipitate on dialysis and this was separated, extracted twice with phosphate-buffered saline (200 ml) and stored as described for Series A.

Fractionation of collagen-associated molecules from 3M-MgCl₂-soluble cartilage collagen complexes

Fig. 1 outlines the subsequent fractionation scheme carried out on the collagen complexes.

Digestion of 3M-MgCl₂-soluble collagen complexes with purified bacterial collagenase. Portions of Series A and B 3M-MgCl_2 -soluble collagen complex (110 mg dry wt.) were dispersed in 0.05M-Tris-HCl – 0.005M-CaCl_2 – 0.02% (w/v) NaN_3 , pH 7.5 (9.8 ml),

Fig. 1. Scheme for the fractionation of collagen-associated molecules from 3M-MgCl_2 -soluble collagen complexes

and collagenase [0.2 ml of a 0.1% (w/v) solution in the same buffer] was added. The mixtures were incubated at 37°C for 18 h and the undigested material was separated by centrifugation (1000g, 10 min). The soluble digests were dialysed and freeze-dried. The undigested residues were not investigated further, since they represented only a small proportion of material (less than 1 mg).

Chromatography on Sepharose 4B. Portions of collagenase digests (12 mg) were dispersed and dissolved in phosphate-buffered saline (3 ml) by sonication for 15 min followed by incubation at 45°C for 30 min. The solutions were allowed to cool to 4°C and applied to a column (38 cm × 2.5 cm; void volume 80 ml) of Sepharose 4B [Pharmacia (G.B.) Ltd., London W.5, U.K.] equilibrated at 4°C. The column was eluted with phosphate-buffered saline, at 12 ml/h, and fractions of volume 4.8 ml were collected. The eluates were monitored at 230 nm and the fractions under each peak of absorption were pooled, dialysed and freeze-dried to give fraction I and fraction II for each series of digests. The monosaccharides present in fraction I were analysed by g.l.c.

Equilibrium-density-gradient centrifugation. Centrifugations were carried out in an MSE Superspeed 65 preparative ultracentrifuge with MSE angle rotor 59592 or 59594. A sample solution, containing 8 mg of fraction I from Sepharose 4B chromatography, was adjusted to 4 M with respect to guanidinium chloride and initial density 1.56 g/ml by the addition of solid CsCl (10 g) and guanidinium chloride (6.4 g). Duplicate volumes of 8 ml were centrifuged at 100000g for 80 h at 18°C. The contents of the tubes were withdrawn as six approximately equal fractions and duplicate fractions were pooled. The density of each fraction was determined by weighing 1 ml portions, and the absorption of each fraction was measured at 280 nm before dialysis. The hexuronic acid content was determined after each dialysed fraction had been made up to 10 ml with distilled water. The remaining solutions were freeze-dried and stored at -20°C.

Preparation of proteoglycan subunit, proteoglycan aggregate and link-protein fraction

Freshly shredded nasal cartilage (20 g) was prepared as described above and extracted for 18 h at 4°C with phosphate-buffered saline (240 ml) followed by 4 M-guanidinium chloride-0.05 M-sodium acetate, pH 5.8 (250 ml) for 72 h at 4°C. Soluble extracts were separated by centrifugation at 2000g for 30 min at 4°C. The phosphate-buffered-saline extract was discarded, but the 4 M-guanidinium chloride extract was dialysed, freeze-dried and stored at -20°C until required for the preparation of proteoglycan subunit, proteoglycan aggregate and link-protein fraction.

Proteoglycan subunit and link-protein fraction. The preparations were carried out by both one-step and two-step equilibrium-density-centrifugation methods of Hascall & Sajdera (1969). In the first method proteoglycan subunit 1 was prepared directly from extract solution (4 M-guanidinium chloride-0.05 M-sodium acetate, pH 5.8) after addition of CsCl to give a density of 1.50 g/ml. Centrifugation was carried out at 100000g for 40 h at 18°C and proteoglycan subunit 1 was isolated from the bottom one-quarter of the gradient. In the second method proteoglycan subunit 2 was obtained by centrifugation of an extract solution (initial density having been adjusted to 1.83 g/ml by addition of solid CsCl) in 0.4 M-guanidinium chloride-0.05 M-sodium acetate, pH 5.8, at 100000g for 60 h at 18°C. The bottom one-third of the gradient was isolated and dialysed into 4 M-guanidinium chloride-0.05 M-sodium acetate, pH 5.8, and solid CsCl was added to bring the density to 1.52 g/ml. Centrifugation was carried out at 100000g for 40 h at 18°C and the top and bottom quarters of the gradient were isolated to give link-protein fraction and proteoglycan subunit 2 respectively.

Proteoglycan aggregate. To prepare proteoglycan aggregate, a recombination experiment was carried out with proteoglycan subunit 1 (70 mg) and the top one-eighth of the gradient (10 mg) obtained from the first CsCl-4 M-guanidinium chloride centrifugation described above. The solution was adjusted by addition of solid CsCl to give an initial density of 1.71 g/ml, 0.4 M with respect to guanidinium chloride and buffered with 0.05 M-sodium acetate, pH 5.8. Centrifugation was performed at 100000g for 40 h at 18°C and proteoglycan aggregate was isolated from the bottom one-quarter of the gradient.

Proteoglycan subunit, proteoglycan aggregate and link-protein fraction were dialysed, freeze-dried and stored at -20°C. Samples were subjected to polyacrylamide-gel electrophoresis.

Interaction of non-collagenous molecules with native collagen

The method of Steven & Jackson (1967) was used to prepare acid-soluble calf skin tropocollagen. Materials to be allowed to interact with this collagen were dissolved in 0.06 M-sodium acetate, pH 4.8, and added to collagen (7 mg) dissolved in the same buffer. The volume of the solution was made up to 3.5 ml with 0.06 M-sodium acetate, pH 4.8. The mixture was stirred gently for 15 h at 4°C and subjected to CM-cellulose chromatography.

CM-cellulose chromatography. Samples for chromatography (Piez *et al.*, 1963) were denatured at 50°C for 45 min. Insoluble material was removed by centrifugation at 1000g for 10 min, and the supernatant was heated for a further 15 min (50°C) and

Table 1. Chemical analyses of material obtained at various stages of fractionation of 3M-MgCl₂-soluble collagen complexes

Values for hydroxyproline and hexuronic acid are expressed as a percentage of dry wt.; n.d., not determined. A and B refer to the two different extraction procedures used.

		3M-MgCl ₂ -soluble collagen complex	Collagenase digest	Sephacrose 4B chromatography	
				I	II
A	Dry weight (mg)	110	48.5	20.3	5.4
	Hydroxyproline	4.4	1.3	0	<1
	Hexuronic acid	n.d.	10.0	26.0	0
B	Dry weight (mg)	110	35.5	16.5	13.2
	Hydroxyproline	3.0	1.0	0	1.5
	Hexuronic acid	n.d.	10.0	19.6	3.2

Fig. 2. Elution of collagenase digests of 3M-MgCl₂-soluble collagen complexes from Sepharose 4B

Portions (12mg) of digests from series A (a) and series B (b) 3M-MgCl₂-soluble collagen complexes were chromatographed at 4°C on a column (38cm×2.5cm) in phosphate buffered saline at a flow rate of 12ml/h. Effluent was monitored at 230nm and fractions of volume 4.8ml were collected.

applied to a column (15cm×1cm) of CM-cellulose (CM-52; Whatman Biochemicals, Maidstone, Kent, U.K.) equilibrated at 42°C with 0.06M-sodium acetate, pH4.8. The flow rate was 27ml/h and 3.9ml fractions were collected. The eluate was continuously monitored at 230nm with a Beckman DB spectrophotometer, and the absorbance was automatically recorded on a Vitatron lin-log converter. The column was washed with 0.06M-sodium acetate until any forepeak had been eluted and then elution was carried out by using a linear gradient of NaCl between 0 and 0.1M (total volume 240ml). Peaks of absorption were pooled, dialysed and freeze-dried and subjected to polyacrylamide-gel electrophoresis.

Results and Discussion

Preparation of 3M-MgCl₂-soluble collagen complexes and their digestion with bacterial collagenase

The hydroxyproline contents of 3M-MgCl₂-soluble collagen complexes obtained by series A and B are shown in Table 1. It can be calculated that the collagen contents lie between 30 and 50%. Polyacrylamide-gel electrophoresis (Plate 1a) of the collagen complexes showed characteristic α_1 and α_2 bands of identical mobility with the bands observed in a preparation of type I collagen (calf skin acid-soluble tropocollagen). It is therefore concluded that the collagen complexes solubilized by 3M-MgCl₂

EXPLANATION OF PLATE I(a)

Electrophoretic separations of fractions obtained from the 3M-MgCl₂-soluble collagen complexes

Electrophoresis was carried out by the method of Furthmayr & Timpl (1971) on 5% polyacrylamide gels. Portions (20 μ l) of sample solution (2.5%, w/v) were electrophoresed for 4 h at 6 mA/tube, and the gels were stained with Coomassie Blue. Cathode is at the top, anode is at the bottom. Gels 1 and 10, acid-soluble calf skin tropocollagen reference sample; gels 2 and 9, 3 M-MgCl₂-soluble collagen complexes; gels 3 and 8, soluble collagenase digests; gels 4 and 7, Sepharose 4B-included material II; gel 6, Sepharose 4B-excluded material I; gel 5, link protein prepared by the method of Hascall & Sajdera (1969) as described in the Methods section. Gels 2-4 represent fractions obtained from Series A, gels 7-9 show fractions from series B. Gel 6 shows material from both series A and B (identical gel in both cases).

EXPLANATION OF PLATE I(b)

Density-gradient ultracentrifugation of peak I from Sepharose 4B separation of collagenase digests

The centrifugation was carried out at 100000g for 80h in the presence of 4M-guanidinium chloride at 18°C. The initial density of the solution was 1.56g/ml. $\circ$, Density; Δ , hexuronate; $\square$, protein. Electrophoretic separations of material obtained from each fraction are shown. Electrophoresis was carried out by the method of Furthmayr & Timpl (1971) under the conditions described for Plate 1(a).

EXPLANATION OF PLATE 2

Elution from CM-cellulose of acid-soluble calf skin tropocollagen allowed to interact with link protein and proteoglycan from 3M-MgCl₂-soluble collagen complexes

CM-cellulose chromatography was carried out at 42°C by the method of Piez *et al.* (1963). Interaction mixtures were denatured at 50°C for 1 h and applied to a column (15 cm × 1 cm) of CM-cellulose equilibrated with 0.06 M-sodium acetate, pH 4.8. Elution was carried out at 42°C at a rate of 27 ml/h with a linear gradient of NaCl (0–0.1 M) in the above buffer. Effluent was monitored at 230 nm, and fractions of volume 3.9 ml were collected. Electrophoretic separations on 5% polyacrylamide gels, of materials under the peaks, were carried out by the method of Furthmayr & Timpl (1971) under the conditions described for Plate 1(a). (a) Control sample of acid-soluble calf skin tropocollagen (7 mg). (b) Acid-soluble calf skin tropocollagen (7 mg) allowed to interact with link protein (1.5 mg). Precipitate obtained after denaturation at 50°C (<0.5 mg) was subjected to gel electrophoresis (gel 5). Gel 1, reference sample of acid-soluble calf skin tropocollagen; gels 2 and 3 are as indicated on the chromatogram; gel 4 is forepeak material. (c) Acid-soluble calf skin tropocollagen (7 mg) allowed to interact with proteoglycan (1.5 mg). Precipitate obtained after denaturation at 50°C (4 mg) was subjected to gel electrophoresis (gel 4). Gel 1, reference sample of acid-soluble calf skin tropocollagen; gel 2 is as indicated on the chromatogram; gel 3 is forepeak material. In all cases forepeaks contained negligible amounts of material, and are not shown on the chromatograms.

EXPLANATION OF PLATE 3

Elution from CM-cellulose of acid-soluble calf skin tropocollagen allowed to interact with increasing amounts of proteoglycan subunit 1

CM-cellulose chromatography was carried out at 42°C on denatured interaction mixtures as described in the Methods section and for Plate 2. Electrophoretic separations on 5% polyacrylamide gels were performed under the conditions described for Plate 1, by the method of Furthmayr & Timpl (1971). (a) Control sample of acid-soluble calf skin tropocollagen (7mg). (b) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit (0.35mg). (c) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit (0.7mg). Precipitate obtained after denaturation at 50°C was washed from the interaction vessel with sample buffer for polyacrylamide-gel electrophoresis (gel 2). Gel 1, reference sample of acid-soluble calf skin tropocollagen; gel 3 is as indicated on the chromatograph. (d) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit (1.0mg). (e) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit (1.5mg). Precipitate obtained after denaturation at 50°C (3mg) was subjected to polyacrylamide-gel electrophoresis (gel 4). Gel 1, reference sample of acid-soluble calf skin tropocollagen; gel 3 is as indicated on the chromatograph; gel 2 is forepeak material.

EXPLANATION OF PLATE 4

Elution from CM-cellulose of acid-soluble calf skin tropocollagen allowed to interact with hyaluronic acid and chondroitin sulphate

CM-cellulose chromatography was carried out at 42°C on denatured interaction mixtures as described in the Methods section and for Plate 2. Electrophoretic separations on 5% polyacrylamide gels were performed under the conditions described for Plate 1, by the method of Furthmayr & Timpl (1971). (a) Control sample of acid-soluble calf skin tropocollagen (7 mg). (b) Acid-soluble calf skin tropocollagen (7 mg) allowed to interact with hyaluronic acid (0.35 mg). Precipitate obtained after denaturation (1.5 mg) was subjected to gel electrophoresis (gel 2). (c) Acid-soluble calf skin tropocollagen (7 mg) allowed to interact with chondroitin sulphate (0.35 and 1.0 mg). Precipitates obtained (2.0 and 4.0 mg respectively) were subjected to gel electrophoresis (gels 3 and 4 respectively). Gel 1 is a reference sample of acid-soluble calf skin tropocollagen.

consist predominantly of type I collagen. After digestion with collagenase, the soluble digest still contained non-diffusible hydroxyproline, but polyacrylamide-gel electrophoresis established that no α , β or γ components were present (Plate 1a). Chemical analysis (Table 1) showed that approx. 90% of the hydroxyproline had been removed by digestion and dialysis.

Fractionation of collagenase digests

Chromatography on Sepharose 4B resulted in the separation of material in the collagenase digests into excluded (fraction I) and included (fraction II) peaks (Fig. 2). Both fractions were heterogeneous when examined by polyacrylamide-gel electrophoresis (Plate 1a). Fraction I preparations exhibited a two-banded system, and were associated with hexuronic acid (Table 1), which accounted for most of the hexuronic acid in the original digest. On polyacrylamide-gel electrophoresis, fraction II preparations were found to contain the remaining bands present in the original digests. Residual hydroxyproline was present in fraction II, and that from series B also had a small amount of hexuronic acid (Table 1). Thus the fractions II were considered to include degradation products from collagenase digestion. The fraction I samples, obtained from the two different preparations, were judged to be identical, comprising material containing hexuronic acid together with two proteins. The proteins were identical in their behaviour on polyacrylamide-gel electrophoresis (Plate 1a) with the glycoprotein-link system being obtained by centrifugation of proteoglycan aggregate in a CsCl-guanidinium chloride gradient, by the method of Hascall & Sajdera (1969) and Hascall & Heinegard (1974a). Separation of the protein components from the material containing hexuronic acid was achieved (Plate 1b) by equilibrium-density-gradient centrifugation in CsCl-4M-guanidinium chloride (Hascall & Sajdera, 1969). The presence of a proteoglycan subunit link-protein type of aggregate was thus indicated.

Analysis by g.l.c.

Fraction I was subjected to g.l.c. after methanolysis. The major monosaccharides were glucuronic acid and galactosamine and there were smaller amounts of glucosamine and galactose. The galactosamine/glucosamine ratio was 4.7:1, which indicated that chondroitin sulphate was the main glycosaminoglycan present.

Preparation of proteoglycan subunit, proteoglycan aggregate and link-protein fraction

After equilibrium-density-gradient centrifugations of 4M-guanidinium chloride extracts of bovine nasal

cartilage, plots of protein, hexuronic acid and density against fraction number were as expected from the work of Hascall & Sajdera (1969). When link-protein fraction was subjected to polyacrylamide-gel electrophoresis, the link protein showed a characteristic two-banded system, also shown by fraction I (Plate 1a). The two bands corresponded to molecular weights of approx. 50000 and 60000, which is in reasonable agreement with the results of Hascall & Heinegard (1974a). Samples of proteoglycan subunit and aggregate gave heavily staining bands at the surface of the gels, indicating non-penetration by high-molecular-weight molecules, presumably the proteoglycans. Photographs of these gels are not shown.

Investigation of interactions with collagen by chromatography on CM-cellulose

Chromatography of calf skin acid-soluble collagen on CM-cellulose gave the expected pattern of α_1 , β_{11} , β_{12} and α_2 peaks (Plate 2a). The criteria used to detect an interaction with this collagen were (a) the formation of an insoluble precipitate after the interaction and denaturation steps, (b) an alteration in the chromatogram profile when compared with the control run, and (c) confirmation of these changes by polyacrylamide-gel electrophoresis.

Both the unfractionated collagenase digests of the 3M-MgCl₂-soluble collagen complexes and fractions I from Sepharose 4B gave positive interactions. Fractions II, however, merely gave a characteristic collagen trace after incubation with collagen. When fraction I was fractionated in a CsCl-4M-guanidinium chloride density gradient, the dense material from the bottom of the gradient (proteoglycan subunit) interacted with collagen (Plate 2c), whereas the link-protein fraction had little or no effect at an equivalent concentration (Plate 2b).

When various amounts of proteoglycan subunit 1 were allowed to interact with a fixed amount of collagen (7mg), the results shown in Plate 3 were obtained. There appeared to be a preferential combination with the β_{12} and α_2 components. This selectivity was maximum when 0.7mg of proteoglycan subunit was used, and all β_{12} and α_2 components appeared in the precipitate. When the amount of proteoglycan subunit was increased to 1.5mg, the result was analogous to that shown in Plate 2(c).

The interaction of proteoglycan aggregate (0.7mg) with collagen is also shown (Fig. 3). The result was similar to that obtained with proteoglycan subunit (0.7mg). It should be pointed out that Hascall & Sajdera (1969) failed to show by analytical ultracentrifugation methods that the proteoglycan aggregate fraction is ever completely aggregated, even under ideal conditions (above pH 5.6). The differences between proteoglycan subunit preparations (1 and 2) on interaction with collagen are minimal (Fig. 3).

Fig. 3. Elution from CM-cellulose of acid-soluble calf skin tropocollagen allowed to interact with proteoglycan subunits 1 and 2 and proteoglycan aggregate

CM-cellulose chromatography was carried out at 42°C on denatured interaction mixtures as described in the Methods section and for Plate 2. (a) Control sample of acid-soluble calf-skin tropocollagen (7mg). (b) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit 1 (0.7mg). (c) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan subunit 2 (0.7mg). (d) Acid-soluble calf skin tropocollagen (7mg) allowed to interact with proteoglycan aggregate (0.7mg). In cases (b)–(d), precipitates obtained after denaturation at 50°C were not investigated further.

The interactions of chondroitin sulphate (1.0mg and 0.35mg) and hyaluronic acid (0.35mg) with collagen (7.0mg) were investigated. Hyaluronic acid was tested, since it has been shown to be one of the link factors in proteoglycan aggregate (Hardingham & Muir, 1973). CM-cellulose chromatography showed that, at 1mg, chondroitin sulphate precipitated all the collagen (Plate 4). When 0.35mg of chondroitin sulphate was used, the areas of the peaks

on the chromatogram were decreased (Plate 4), and some collagen was precipitated. A similar result was obtained with hyaluronic acid (0.35mg), except that the extent of the decrease in peak areas was not as great as that observed for chondroitin sulphate (Plate 4). Neither glycosaminoglycan appeared to interact preferentially with α_2 or β_{12} chains, since all peak areas seemed to be decreased by the same proportion.

General Discussion

Polyacrylamide-gel electrophoresis of the 3M-MgCl₂-soluble collagen complexes indicated that collagen constituents were predominantly type I (Plate 1). The question of whether mature cartilage contains any type I collagen has been discussed in several reports. Miller & Lunde (1973) inferred that only type II collagen was present in the 4M-guanidinium chloride-insoluble residue from bovine nasal cartilage, and bovine and human articular cartilage, and Strawich & Nimni (1971) isolated type II collagen after papain digestion of bovine articular cartilage. However, both type I and II collagens have been detected in several lathyrctic chick cartilages (Glimcher *et al.*, 1969; Miller & Matukas, 1970; Trelstad *et al.*, 1970; Miller, 1971), and in the annulus fibrosus of mammalian intervertebral disc (Eyre & Muir, 1975). Seyer & Vinson (1974) reported that both type I and II collagen are synthesized simultaneously in embryonic and post-hatch chick keel and articular cartilage, but that the proportion of type I falls as the cartilage develops. The repeated observation that our preparations contained type I collagen, as judged by polyacrylamide-gel electrophoresis, indicates that this a genuine but minor component of bovine nasal cartilage, and appears to be selectively extracted by 3M-MgCl₂.

Interaction studies showed that proteoglycan, and not link components, bind to type I collagen. The proteoglycan had the greatest affinity for the α_2 and β_{12} components, and by increasing the amount of proteoglycan the interaction could be extended to include the α_1 and β_{11} constituents. Thus the isolation of a complex of type I collagen with proteoglycan, and the interaction *in vitro* between proteoglycan subunit and type I collagen, suggested that such interactions may be of importance in cartilage.

The stronger binding of proteoglycan to α_2 compared with α_1 chains could be due to either the more basic nature of the α_2 chain (Eastoe, 1967), or a more specific effect in which the basic residues are better placed in the primary sequence of the α_2 chain than the α_1 chain to form favourable electrostatic interactions with the glycosaminoglycan side chains of the proteoglycan. The latter explanation is supported by the observation that the interaction of

chondroitin sulphate with tropocollagen involves α_1 and α_2 chains equally. Hascall & Sajdera (1970) showed that the glycosaminoglycan side chains of proteoglycan subunits are mainly chondroitin sulphate. In other words, the ordered spatial arrangement forced on the glycosaminoglycan chains by their attachment to protein core allows them to engage in electrostatic bonding with basic residues in the α_2 chain, but the basic residues in the α_1 chain are not so suitably placed, so that a poorer 'overlap' of glycosaminoglycan chains and basic residues occurs.

The basis of the method for investigating interactions was CM-cellulose chromatography of the denatured interaction mixture. The advantage of this method is that only firmer aggregates will remain intact after incubation at 50°C and they can then be isolated as precipitates or in the fraction that passes directly through the column. Non-interacting macromolecules, e.g. link protein, do not alter the chromatogram profile significantly (Plate 2). The study of interactions on the acid side of physiological pH is valid, since neither the sulphate monoesters of the proteoglycan nor the positively charged side groups of collagen, both of which are involved in binding (Podrazky *et al.*, 1971), are affected, although carboxyl groups on both molecules will be partially neutralized. The finding that proteoglycan from bovine nasal cartilage interacts with collagen adds support to reports already published (Obrink & Wasteson, 1971; Lowther & Natarjan, 1972) on the interaction of proteoglycans with collagen. However, the use of CM-cellulose chromatography allows the mode of interaction to be probed in greater detail than is possible with any method previously described.

The crucial question raised by these studies is how the affinity of proteoglycan subunit for $\alpha_{1(II)}$ compares with its affinity for $\alpha_{1(I)}$ and α_2 . Bearing in mind that type I collagen must be a minor constituent of mature nasal cartilage, the relevance of our results to a true concept of the intercellular matrix is impossible to assess until we have carried out parallel experiments with type II collagen. The greater proportion of type I collagen in developing cartilage has been interpreted by Seyer & Vinson (1974) as indicating that type I collagen could have an important role in initial cartilage matrix deposition.

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