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A novel low-molecular-weight chondroitin sulphate proteoglycan isolated from cartilage

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Proteoglycans were isolated from cartilage by extraction with 4 M-guanidinium chloride followed by direct centrifugation in 4 M-guanidinium chloride/CsCl at a low starting density, 1.34 g/ml. *N*-Ethylmaleimide was included in the extraction solvent as a precaution against contamination of proteoglycans with unrelated proteins mediated by disulphide exchange. A novel, discrete, low-buoyant-density proteoglycan (1.40–1.35 g/ml) was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Its proteoglycan nature was revealed by the shift in the molecular size observed on gel electrophoresis after treatment with chondroitinase ABC. The core protein was monodisperse. The proteoglycan was further purified by gel chromatography with and without addition of hyaluronate. The proteoglycan constitutes less than 2% (by weight) of the total extracted proteoglycans and is not capable of interacting with hyaluronate. The same proteoglycan was purified in larger quantities by sequential associative and dissociative CsCl-density-gradient centrifugation, zonal rate sedimentation in a sucrose gradient and gel chromatography on Sepharose CL-4B. The pure proteoglycan had a molecular weight of 76 300 determined by sedimentation-equilibrium centrifugation and an apparent partial specific volume of 0.59 ml/g. It contained about 25% protein (of dry weight) and had remarkably high contents of leucine and cysteine as compared with other proteoglycans. The proteoglycan contained two to three large chondroitin sulphate chains and some oligosaccharides.

Proteoglycans are major constituents of the cartilage intercellular matrix. A proteoglycan molecule has a central protein core to which polysaccharide side chains are attached via their reducing terminals. The cartilage proteoglycan, in particular, contains a very large number of chondroitin sulphate and keratan sulphate chains, attached to different regions of the protein core (Heinegård & Axelsson, 1977). Another region of the core protein, located at one end, has a structure allowing specific interaction with hyaluronic acid, the hyaluronic acid-binding region (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). The core protein is very polydisperse, having a molecular weight of 200000–300000 (Hascall & Riolo, 1972; Hascall & Heinegård, 1974*a*). The intact proteoglycan monomers are also very polydisperse (Heinegård, 1977), with molecular weights ranging from less than one million to several millions, with an average of about 2.5×10^6 (Hascall & Sajdera, 1970).

The hyaluronic acid-binding region mediates a specific interaction between the proteoglycan and hyaluronic acid (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*a*,*b*; Hardingham *et al.*, 1976). Several proteoglycan monomers can bind to one molecule of hyaluronic acid, forming an extremely large proteoglycan aggregate. The aggregating proteoglycans, which contain the hyaluronic acid-binding region, constitute about 85% of the total extractable proteoglycans in cartilage. Another population, the non-aggregating proteoglycans, representing about 10%, cannot interact with hyaluronic acid (Heinegård & Hascall, 1979). These molecules do not contain the hyaluronic acid-binding region, although they contain more protein and less chondroitin sulphate (Heinegård & Hascall, 1979).

There have been reports of yet another population of proteoglycans of similar size to the non-aggregating proteoglycans, but containing little protein and mainly chondroitin sulphate (Mason & Mayes, 1973; Pearson & Mason, 1977). These proteoglycans, however, were isolated by techniques in which proteolysis was not minimized. A very slowly sedimenting proteoglycan of low buoyant density in CsCl density gradients has been

demonstrated as a sulphate-labelled component in extracts from embryonic cartilages and cell cultures (Kimata *et al.*, 1978). Swann *et al.* (1979) fractionated an extract of articular cartilage to subfractions containing molecules of very different sizes. One fraction contained small proteoglycans of a low buoyant density.

The present investigation was initiated in an attempt to design a procedure to isolate all extractable proteoglycans, under conditions preventing activity of degrading enzymes. It was considered important to isolate also proteoglycans of low buoyant density not bound into aggregates and therefore not recovered in the normal isolation procedure.

Materials and methods

Bovine nasal cartilage was obtained at the abattoir within a few minutes after slaughter. The cartilage was immediately carefully cleaned from surrounding tissue and perichondrium and sliced with a Surform blade. The cartilage slices were stored at −60°C until extraction. Cartilage slices were extracted for 24h at 3°C with 12vol. of 4M-guanidinium chloride/50mM-sodium acetate buffer, pH5.8, containing the proteinase inhibitors 5mM-benzamidine hydrochloride, 0.1M-6-aminohexanoic acid and 10mM-EDTA (Oegema *et al.*, 1975). In addition, the extraction solvent contained 4mM-*N*-ethylmaleimide to prevent disulphide exchange and to inhibit any thiol proteinase. The extract was then filtered through Celite (BDH Chemicals, Poole, Dorset, U.K.).

CsCl-density-gradient centrifugation

The extract, containing about 6mg of proteoglycan/ml, was adjusted to a density of 1.34g/ml by addition of solid CsCl. It was then centrifuged in an MSE 8×25ml angle rotor (22ml per tube) at 35000rev./min (95000*g*, $r_{av.}$ 6.886cm) for 60h at 18°C. By use of a tube piercer the tubes were emptied from the bottom to yield 11 fractions of volume 2ml.

In other experiments identical extracts were dialysed against 10vol. of 50mM-sodium acetate buffer, pH5.8, containing the proteinase inhibitors listed above. After associative centrifugation (starting density 1.64g/ml) as described elsewhere (Heinegård & Hascall, 1979), the top three-quarters of the tubes (A_{top} fraction) were recovered and dialysed against an equal volume of 8M-guanidinium chloride. The density was adjusted to 1.35g/ml by adding a small amount of solid CsCl. After centrifugation for 60h as described above, the tubes were emptied in 2ml fractions.

Densities of fractions were measured by using a 200μl constriction pipette. Samples (25μl) were removed for determination of uronic acid. Other samples were taken for electrophoresis on sodium dodecyl sulphate/polyacrylamide gels as described below. Care was taken so that each sample electrophoresed represented an equal portion of the original fraction. The fractions from the CsCl gradients were pooled and extensively dialysed against sodium acetate and distilled water and were finally freeze-dried.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Samples from the various fractionation procedures were dialysed into 20mM-Tris/HCl buffer, pH8.0. Equal portions were incubated with 0.002 unit of chondroitinase ABC (Miles Laboratories, Elkhart, IN, U.S.A.)/100μl for 5h at 37°C and without addition of enzyme. Before electrophoresis an equal volume of 2-fold-concentrated upper-reservoir buffer containing 10% (w/v) sucrose, 0.01% (w/v) EDTA and 10% (v/v) 2-mercaptoethanol was added and the samples were incubated for 2h at 37°C. In some cases the 2-mercaptoethanol was omitted. Samples containing 10–50μg of protein were electrophoresed on polyacrylamide gels in the buffer system of Neville (1971). The gels were T = 8.0% and C = 2.5%, where T is the percentage (w/v) of total monomer and C is the amount of *NN'*-methylenebisacrylamide expressed as fraction (%, w/w) of total monomer (Neville, 1971). Gels were stained with Kenacid Blue R (BDH Chemicals).

In a separate experiment the purified proteoglycan was digested with chondroitinase ABC (0.004 unit/mg) and electrophoresed on polyacrylamide gels (T = 6–14%, C = 1%). Reference proteins (high-molecular-weight and low-molecular-weight standard proteins; Pharmacia Fine Chemicals, Uppsala, Sweden) were electrophoresed in an identical manner and the dependence of the relative mobility on the polyacrylamide concentration was determined, essentially as described by Banker & Cotman (1972).

Sucrose-gradient zonal rate sedimentation

The material that was recovered from the top fraction (A_{top}) of an associative CsCl density gradient (without guanidinium chloride), and then centrifuged in a dissociative CsCl density gradient (with guanidinium chloride) and recovered in the bottom fraction (A_{top}-D1 fraction), was extensively dialysed against water. Tris/HCl buffer (0.1M), pH7.5, was added to give a final concentration of 1mM. The samples (2ml) were layered on top of 14ml of a 10–30% (w/v) linear sucrose gradient in 1mM-Tris/HCl buffer, pH7.5. After centrifugation at 50000rev./min (190000*g*, $r_{av.}$ 6.886 cm) for 10h

in an MSE 8 × 25 ml angle rotor the tubes were emptied and the absorbance of the effluent at 206 nm was recorded as described elsewhere (Franzén *et al.*, 1981).

Column chromatography

Columns (0.8 cm × 140 cm and 2.0 cm × 140 cm) of Sepharose CL-4B were eluted with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and fractions of volume 1.5 and 10 ml respectively were collected. About 2.5 mg and 50 mg of sample respectively were applied to the columns. A Sepharose 4B column (1.6 cm × 140 cm) was eluted with 0.5 M-sodium acetate buffer, pH 5.8, and 4.9 ml fractions were collected. Approx. 15 mg of sample was applied. Samples to be chromatographed were dissolved in 4 M-guanidinium chloride and dialysed against the solvent used for chromatography. A column (0.6 cm × 180 cm) of Bio-Gel P-30 was eluted with 0.25 M-pyridinium acetate buffer, pH 6.5. Fractions of volume 0.8 ml were collected.

Enzymic treatment

To test for the presence of dermatan sulphate and chondroitin sulphate, equal samples of the purified proteoglycan were dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, and digested with 0.05 unit of chondroitinase AC-II and chondroitinase ABC/mg respectively for 6 h at 37°C. The samples were separately chromatographed on Bio-Gel P-30.

Samples were digested for 5 h at 60°C with papain (2 × crystallized and containing about 25 mg/ml of protein; Sigma Chemical Co., St Louis, MO, U.S.A.) (1 μl/mg of proteoglycan) in 50 mM-sodium phosphate buffer, pH 6.5, containing 10 mM-EDTA and 5 mM-cysteine hydrochloride. The digests were then either chromatographed on Sephadex G-200 to reveal the size of the side chains or chromatographed on ECTEOLA-cellulose to isolate the glycosaminoglycans (Axelsson & Heinegård, 1975).

Analytical ultracentrifugation

Molecular weights were determined by sedimentation-equilibrium centrifugation by the high-speed method of Yphantis (1964). The sample was dissolved in and extensively dialysed against 4.0 M-guanidinium chloride (Ultrapure; Schwarz–Mann, Orangeburg, NY, U.S.A.)/5 mM-Tris/HCl buffer, pH 7.0. An MSE Centriscan analytical ultracentrifuge was used and the distribution of the material at equilibrium was determined both by using the schlieren system and by using the photoelectric scanner and a 280 nm interference filter. Apparent partial specific volume of the proteoglycan in 4 M-guanidinium chloride was determined by using the ^2H_2O method (Thomas & Edelstein, 1971). The apparent molecular weight of the proteoglycan in guanidinium chloride/^2H_2O was corrected by the factors 1.0155 for the protein and 1.0158 for the glycosaminoglycan part to compensate for exchangeable hydrogen atoms being replaced by deuterium.

Sedimentation coefficients were determined by using the MSE Centriscan analytical ultracentrifuge. Samples were centrifuged in buffered 4.0 M-guanidinium chloride (as above) at 59000 rev./min. Scans at 280 nm were taken at regular intervals by using the photoelectric scanner. Sedimentation coefficients were calculated in accordance with the MSE manual (Technical Publication, Supplement no. 73), corrected to 20°C and water and extrapolated to zero concentration.

Chemical methods

Uronic acid contents of fractions from the density gradients and column effluents were determined by the carbazole method (Bitter & Muir, 1962). When columns were eluted with 0.5 M-sodium acetate buffer or 0.25 M-pyridinium acetate buffer, the uronic acid content was determined by an automated carbazole procedure (Heinegård, 1973).

Sialic acid content was determined by the method of Jourdain *et al.* (1971).

Hexosamine contents were determined after hydrolysis of samples in 4 M-HCl at 100°C for 10 h under argon. Amino acid contents were determined after hydrolysis of samples in 6 M-HCl at 110°C for 24 h under argon. The HCl used was of AristaR quality (BDH Chemicals). A Durrum automatic amino acid analyser was used for both hexosamine and amino acid determinations.

Neutral sugar content was determined by ion-exchange chromatography of borate complexes of monosaccharides as described by Walborg & Kondo (1970) and modified by S. Lohmander (personal communication). The sample was hydrolysed in 2 M-trifluoroacetic acid for 2 h at 100°C.

Results and discussion

Isolation of proteoglycans under denaturing conditions

One of the objects of the present work was to develop a method for isolation of all proteoglycans in cartilage under conditions minimizing degradation. Therefore in initial experiments the extract was centrifuged directly in 4 M-guanidinium chloride, avoiding the non-denaturing conditions of reassociation. One problem encountered was that, with the commonly used starting density of 1.5 g/ml, some proteoglycans will under dissociative conditions be recovered in or near the top fraction of the gradient, as is discussed elsewhere (Heinegård, 1977). Therefore we decided to use the much lower starting density of 1.34 g/ml. The density obtained in the bottom fraction, then, is actually about the same

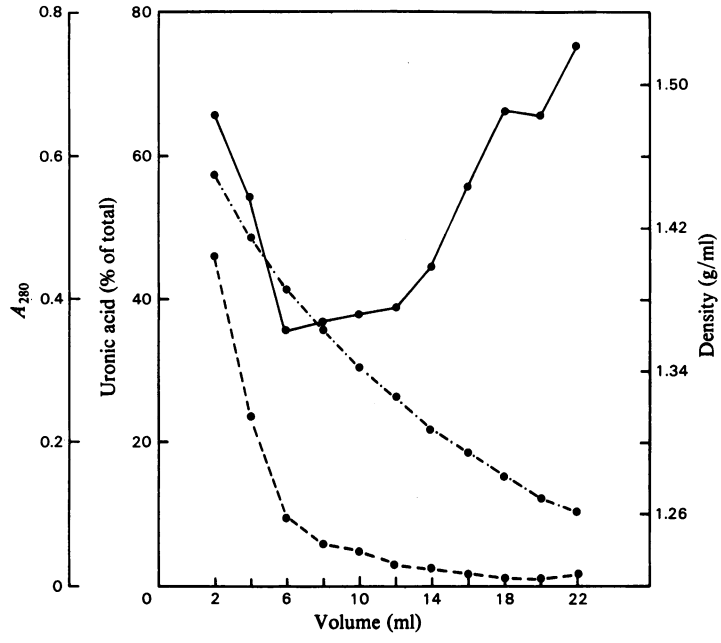

Fig. 1. *Guanidinium chloride (4 M)/CsCl-density-gradient centrifugation (starting density 1.34 g/ml) of cartilage extract*
Experimental details are given in the text. ——, A_{280}; – – – –, A_{530} (carbazole reaction) as percentage of total in the gradient; —·—·—, density.

as that obtained for the top fraction of a gradient with a starting density of 1.52 g/ml. The bottom third of the centrifuge tube contained more than 85% of the uronic acid in the gradient (Fig. 1) and about 80% of the recovered material (by weight).

In order to determine possible contamination with non-proteoglycan proteins, equal portions of the fractions (pooled two and two from the bottom) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without reduction (Figs. 2*a* and 2*b*). No Kenacid-staining protein bands were observed in the bottom two pools analysed, i.e. the bottom 8 ml. It appears, then, that proteoglycans can be purified in good yield by direct CsCl-density-gradient centrifugation of 4 M-guanidinium chloride extracts at low starting density. In preliminary experiments, when extraction was performed, without *N*-ethylmaleimide in the extraction solvent, protein bands were occasionally observed in reduced samples of the bottom fraction. It is possible that proteoglycans may form complexes with other proteins through disulphide exchange, which may be favoured by the denaturing conditions used for extraction. Proteins bound to the proteoglycans will sediment to the bottom of the gradients. Because of such results it was considered important to include *N*-ethylmaleimide to block free thiol groups in all extraction solvents used.

Equal portions of the fractions from the CsCl-gradient centrifugation were digested with chondroitinase ABC to remove chondroitin sulphate side chains. They were then electrophoresed with and without reduction (Figs. 2*c* and 2*d*). The amount of enzyme used did not give any detectable bands. In separate experiments it was shown that the chondroitinase used did not contain detectable proteolytic activity, when [^{3}H]acetylated haemoglobin was used as a substrate. The fraction (D2) obtained from a density of 1.40–1.35 g/ml contained a protein band with a mobility close to that of the link proteins (Keiser *et al.*, 1972; Hascall & Heinegård, 1974*a*). Since the chondroitinase treatment had only removed the chondroitin sulphate side chains, it was considered probable that this band represents the core protein of a low-buoyant-density small proteoglycan. It is noteworthy that the protein core is monodisperse, in contrast with the extreme polydispersity of the high-buoyant-density proteoglycans. In a separate experiment samples of all the fractions of an identical density gradient were digested with chondroitinase ABC and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. The gel patterns showed the core protein as a band with a mobility intermediate between those of the two major link proteins (results not shown).

All the other proteins extracted from the cartilage

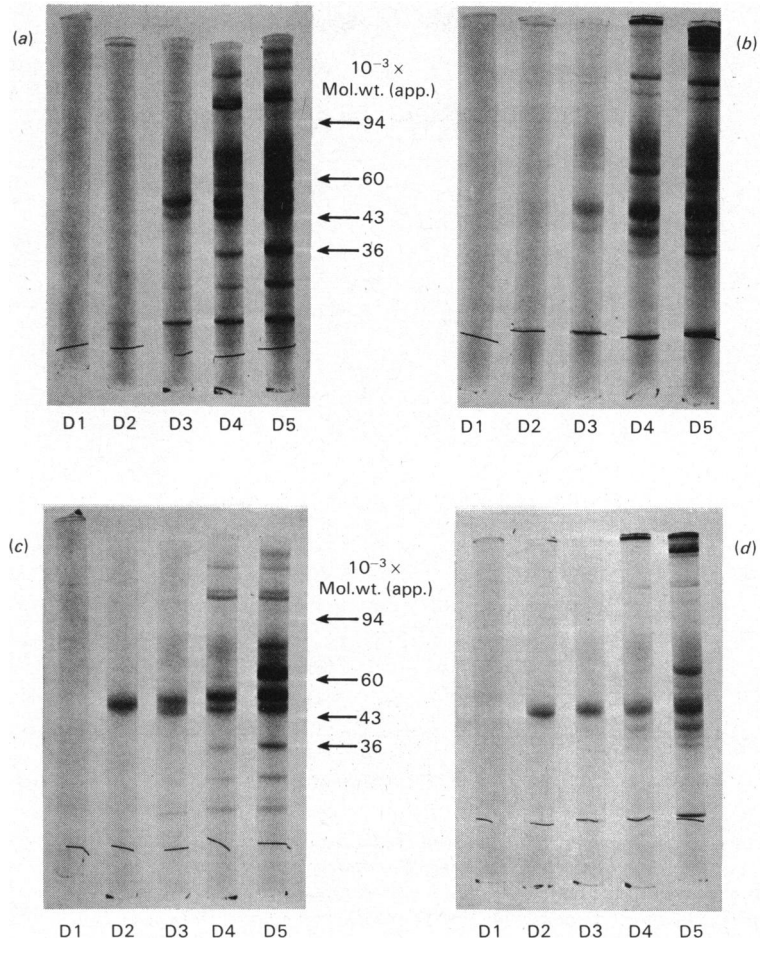

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions (4 ml) from 4 M-guanidinium chloride/CsCl-density-gradient centrifugation*

Experimental details are given in the text. (*a*) Reduced samples; (*b*) non-reduced samples; (*c*) samples digested with chondroitinase, reduced; (*d*) samples digested with chondroitinase, non-reduced.

were also present in the gels. The fact that the link proteins were found as far from the top fractions as in fraction D3 is important. This may be a result of the re-orientation of the gradient in the angle rotor at the end of centrifugation. The matrix protein previously isolated from tracheal cartilage (Paulsson & Heinegård, 1979, 1981) was, however, present only in the top fraction of this nasal-cartilage preparation (Fig. 2). It is possible that the link protein is not at equilibrium at the top of the gradient.

The two fractions that contained the major proportion of the low-buoyant-density proteoglycan (corresponding to D2 fraction in Fig. 2) were pooled, extensively dialysed against sodium acetate buffer and distilled water and then freeze-dried. The material recovered constituted about 6% of the total in the gradient (by weight). The bottom fractions, which contained only high-buoyant-density large proteoglycans, weighed 12.5 times as much as the low-buoyant-density small proteoglycan fraction.

A sample (50 mg; d-D2 fraction) was chromatographed on the preparative Sepharose CL-4B column eluted with 4 M-guanidinium chloride (Fig. 3). Two major peaks were observed. A uronic acid-rich peak was eluted at the void volume, and a protein-rich peak was eluted well included. The effluent fractions were pooled as indicated in Fig. 3. A total of 48 mg was recovered, of which 26 mg was from the protein-rich peak. Samples, representing equal portions of the fractions, were taken for chondroitinase ABC digestion and sodium dodecyl

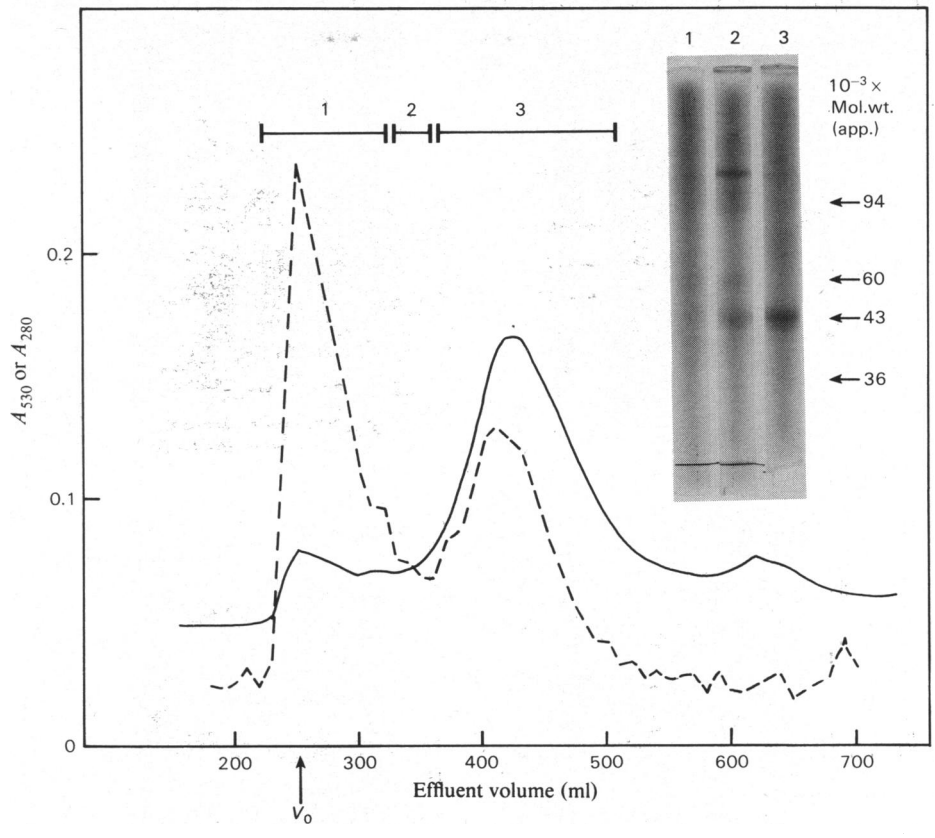

Fig. 3. *Sepharose CL-4B-chromatography (eluent 4 M-guanidinium chloride) of the bottom fraction from 4 M-guanidinium chloride/CsCl-density-gradient centrifugation*

Experimental details are given in the text. ——, A_{280}; – – – –, A_{530} (carbazole reaction). The insert shows the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis patterns of samples (80μg) of the indicated fractions after chondroitinase digestion. V_0, Void volume.

sulphate/polyacrylamide-gel electrophoresis after reduction (Fig. 3). The band corresponding to the low-buoyant-density proteoglycan was observed only in the gel corresponding to the protein-rich included peak (d-D2-4B2 fraction). The material in the void volume, then, probably represents low-buoyant-density proteoglycans of the polydisperse population representing the bulk of the proteoglycans in cartilage.

Preliminary experiments showed that the proteoglycan preparation contained some material that reacted with antibodies directed against the hyaluronic acid-binding region of regular cartilage proteoglycan monomers (results not shown). Molecules containing the hyaluronic acid-binding region are capable of interacting with hyaluronic acid. Therefore it should be possible to shift the elution position of such molecules to the void volume by the addition of very-high-molecular-weight hyaluronic acid, which in itself chromatographs excluded from the gel. A sample (15 mg) of the low-buoyant-density small proteoglycan, purified by Sepharose CL-4B chromatography (d-D2-4B2 fraction), was dissolved in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. After dialysis against 500 vol. of 0.5 M-sodium acetate buffer, pH 5.8, high-molecular-weight hyaluronic acid (Healon, a gift from Pharmacia Fine Chemicals) was added (1%, by weight) and the sample was incubated overnight at 4°C. It was then chromatographed on a column of Sepharose 4B eluted with 0.5 M-sodium acetate buffer, pH 5.8 (Fig. 4). A relatively more-protein-rich peak was eluted in the void volume of the column, and a more-uronic acid-rich component was eluted well included. Fractions were pooled as indicated in Fig. 4, dialysed against sodium acetate buffer and distilled water, and freeze-dried. The included component constituted 52% (w/w) of the material recovered. Equal portions of each fraction were digested with chondroitinase ABC and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. A strongly staining band corresponding

to the low-buoyant-density small proteoglycan was present in the gel containing the sample from the uronic acid-rich included peak. This gel also showed a minor contaminating protein component with a lower mobility (Fig. 4). The molecular weight of the material in the purified proteoglycan fraction was determined by sedimentation-equilibrium centrifugation. After extrapolation to zero concentration, values of 67600 and 70200 were obtained by scanning at 280nm and with the schlieren optics respectively. A value for apparent partial specific volume of 0.59ml/g was used, as discussed below. The lower value obtained with the scanning at 280nm may reflect the presence of a low-molecular-weight protein contaminant. In support, the plots of $\ln c$ versus r^2 were non-linear at the lower concentration range.

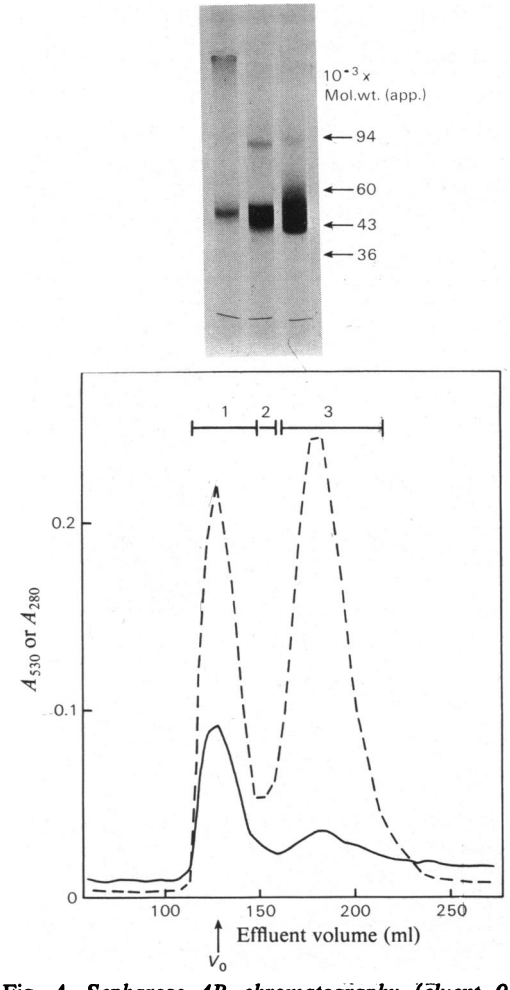

Fig. 4. *Sepharose 4B chromatography (eluent 0.5M-sodium acetate buffer, pH5.8) of the included fraction from dissociative chromatography of the bottom fraction from 4M-guanidinium chloride/CsCl-density-gradient centrifugation (d-D1-4B2 fraction)*

Experimental details are given in the text. ——, A_{280}; – – – –, A_{530} (carbazole reaction). At the top is shown the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis patterns of samples (100μg) of the indicated pools with and without chondroitinase digestion. V_0, Void volume.

Isolation of the small proteoglycan after associative CsCl-density-gradient centrifugation

To purify the proteoglycan further and in larger amounts, an alternative procedure was devised. Cartilage was extracted with 4M-guanidinium chloride, and proteoglycans in the extract were reassociated essentially as described elsewhere (Heinegård & Hascall, 1979), the only difference being that *N*-ethylmaleimide was included in the solvent to prevent disulphide exchange. After associative CsCl-density-gradient centrifugation, the top three-quarters of the tube (A_{top} fraction) contained all of the small proteoglycan and a proportion of other types of cartilage proteoglycans. To separate proteoglycans from proteins, this material was centrifuged under the dissociative conditions of 4M-guanidinium chloride in a CsCl gradient with the starting density of 1.35g/ml. Equal portions of the fractions were then dialysed into water and freeze-dried. Each portion was digested with chondroitinase ABC, reduced and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels (Fig. 5). The bottom pool (6ml; A_{top}-D1 fraction), which contained the major portion of the small proteoglycan, as indicated by the presence of the core protein, was dialysed into water. This material was about 10% (by weight) of the A1

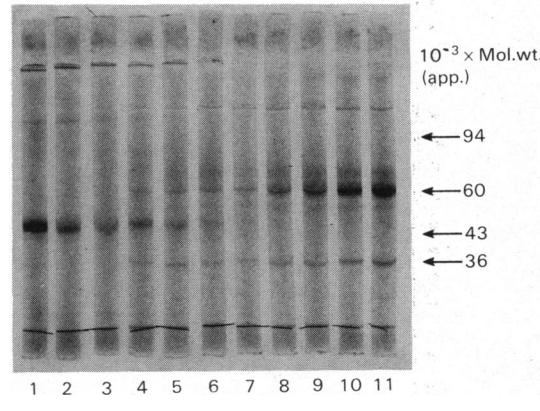

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chondroitinase-digested and reduced fractions from sequential associative–dissociative CsCl-density-gradient centrifugation of cartilage extracts*

For experimental details see the text.

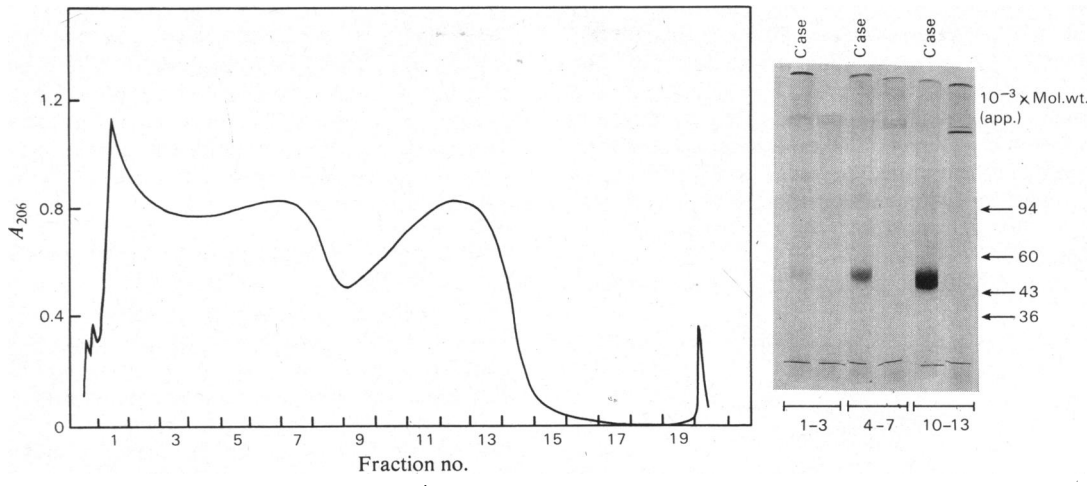

Fig. 6. *Zonal rate centrifugation in 10–30% (w/v) sucrose in 1 mM-Tris/HCl buffer, pH 7.5, of the bottom fraction from sequential associative–dissociative CsCl-density-gradient centrifugation (A_{top}-D1 fraction)*

Experimental details are given in the text. Samples (50 μg) of indicated fractions were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels before and after chondroitinase (C'ase) digestion. ——, A_{206}.

fraction from the same preparation. Samples were subjected to zonal rate centrifugation in sucrose density gradients, to separate the small proteoglycans from any larger cartilage-type proteoglycans present. In preliminary experiments it was shown that optimal separation was obtained when the salt concentration in the gradient was kept as low as possible. The tracing of the absorbance at 206 nm of the tube contents showed at least two major components, as well as the presence of material that had sedimented to the bottom (Fig. 6). Samples of the indicated fractions were recovered and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis before and after chondroitinase-ABC digestion (Fig. 6). The major portion of the small proteoglycan was present in the slowest-sedimenting component (A_{top}-D1-SS2 fraction). The material in this fraction was recovered after dialysis against water and freeze-drying. It contained about 25% (by weight) of all material recovered from the zonal rate centrifugation. This preparation was then dissolved in 4 M-guanidinium chloride and chromatographed on a Sepharose CL-4B column eluted with the same solvent. One component was eluted in the void volume (Fig. 7), and another component was eluted included. The indicated fractions were recovered. The included material (A_{top}-D1-SS2-4B2 fraction) represented about 55% (by weight) of the material applied to the column. Samples of the two pools were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without prior digestion with chondroitinase ABC (Fig. 7). It appears that the included fraction contained only the small proteoglycan. No bands other than

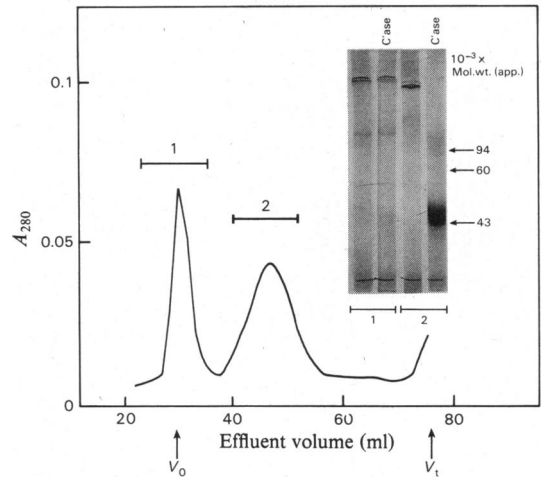

Fig. 7. *Sepharose CL-4B chromatography (eluent 4 M-guanidinium chloride) of the fraction containing the small proteoglycan recovered from zonal rate centrifugation in a sucrose density gradient (cf. Fig. 6)*

Experimental details are given in the text. Samples (50 μg) of indicated fractions were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels before and after chondroitinase (C'ase) digestion. ——, A_{280}. V_0, Void volume; V_t, total volume.

the core protein were observed in the two gels of the included proteoglycan component, indicating purity of the proteoglycan. The excluded fraction contained a minor component with a much lower mobility, which was not affected by chondroitinase

digestion, indicating that it represents a molecule not containing galactosaminoglycans. This component had the same mobility as the impurity of the dissociatively prepared small proteoglycan discussed above. In addition, both the gels contained material not entering the gel, probably high-molecular-weight proteoglycans.

Analysis of the small proteoglycan

The preparation of the small proteoglycan was considered pure and was further analysed. Its molecular weight was determined to be 76300 by sedimentation-equilibrium centrifugation in 4M-guanidinium chloride (Fig. 8). Identical results were obtained by using the schlieren optics and by scanning at 280nm. This value is in agreement with those obtained for the dissociatively prepared proteoglycan (see above). Consequently the molecule can be prepared by a scheme including non-denaturing conditions as well as when maintaining denaturing conditions during the preparation to abolish degradation. The molecular weight represents a lowest value, since molecular weights of polydisperse molecules are usually underestimated when the meniscus-depletion method is used. It should be stressed, however, that the same values were obtained when centrifugation was done at 15000–20000 rev./min, indicating that the effects of the polydispersity were minor. The apparent partial specific volume in 4M-guanidinium chloride was determined to be 0.59ml/g. Owing to the complexity of assessing the partial specific volume of proteoglycans, this value should be considered an estimate. Sedimentation-velocity centrifugation in 4M-guanidinium chloride yielded an $s^0_{20,w}$ value of 3.11S.

The proteoglycan was further characterized (Table 1). Approx. 25% of the dry weight was protein. The amino acid composition was quite different from that of other cartilage proteoglycans (Heinegård & Hascall, 1979). The contents of serine and threonine were low, indirectly indicating a relatively low glycosaminoglycan content. In contrast, the content of leucine was very high and that of cysteine was comparatively high. The amino acid composition is similar to that previously described for a keratan sulphate proteoglycan with a similar molecular weight, which was isolated from bovine cornea (Axelsson & Heinegård, 1978). The proteoglycan had lower contents of hexosamines than did

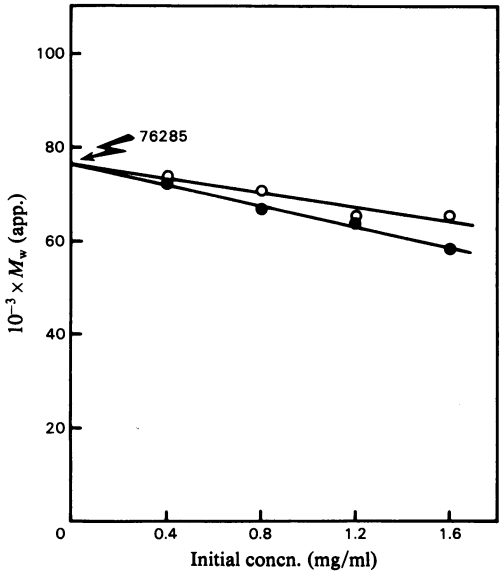

Fig. 8. *Determination of the molecular weight of the small proteoglycan by sedimentation-equilibrium centrifugation*

Experimental details are given in the text. Extrapolation was to zero concentration. ○, Schlieren; ●, scanning at 280nm.

Table 1. *Amino acid and carbohydrate composition of the low-molecular-weight cartilage proteoglycan*

For experimental details see the text.

	Composition	
	(nmol/mg)	(residues/1000 residues)
Aspartic acid	309	129
Threonine	94	39
Serine	158	66
Glutamic acid	234	98
Proline	173	72
Glycine	170	71
Alanine	121	50
Cysteine	45	19
Valine	128	53
Methionine	2	1
Isoleucine	131	55
Leucine	342	143
Tyrosine	66	28
Phenylalanine	81	34
Histidine	86	36
Lysine	154	64
Arginine	107	44
Glucosamine	65	
Galactosamine	841	
Uronic acid	917	
Mannose	33	
Galactose	27	
Fucose	14	
Xylose	42	
Glucose	17	
Sialic acid	Not detected	
Protein (% dry wt.)	25.2	
Hexosamine (% dry wt.)	16.2	
Uronic acid (% dry wt.)	17.8	

other cartilage proteoglycans. Galactosamine was the major hexosamine. In separate experiments the glycosaminoglycans were isolated by ion-exchange chromatography of a papain digest of the proteoglycan (Axelsson & Heinegård, 1975). They contained only galactosamine, indicating that the proteoglycan contained only galactosaminoglycans and that the glucosamine was present in oligosaccharides attached to the protein core. In support, the proteoglycan contained mannose and fucose, not present in the glycosaminoglycan side chains. The low content of galactose may be due to losses during hydrolysis. It is notable, however, that the molar ratio of xylose to galactosamine is much lower than would be expected from the large chondroitin sulphate side chains (discussed below). It is possible that the proteoglycan contains some xylose residues not further substituted with a glycosaminoglycan side chain. The glucose present may be due to some sucrose remaining from the zonal rate centrifugation.

The character of the galactosaminoglycan side chains was determined. One sample of the proteoglycan was digested with chondroitinase AC-II and another identical sample was digested with chondroitinase ABC. The two chromatograms were identical (Fig. 9), and virtually all of the uronic acid was eluted in the total volume of the column. Consequently, the side chains were chondroitin sulphate that could be completely depolymerized by the action of chondroitinase AC.

The chondroitin sulphate was liberated by papain digestion of the proteoglycan and chromatographed on Sephadex G-200 (Fig. 10). The chains were eluted less retarded than a control sample of chondroitin sulphate isolated from a preparation of bovine nasal-cartilage proteoglycan monomer (A1-D1 fraction).

The protein content of the small proteoglycan indicated that the molecular weight of the protein core should be about 20000. This value is considerably lower than the apparent molecular weight observed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the core preparation after chondroitinase-ABC digestion. Therefore the dependence of the electrophoretic mobility of the core protein on the concentration of polyacrylamide was determined over the range 6–14%. By using the theoretical approach of Ferguson (1964), retardation coefficients (K_R) and apparent free electrophoretic mobilities (M_0) could be calculated for the core protein and for a number of reference proteins. A plot of K_R versus molecular weight of reference proteins was linear, and from this relationship an apparent molecular weight of the core protein of the small proteoglycan of 42300 was obtained. Banker & Cotman (1972) have suggested plots of M_0 versus K_R as a sensitive test of ideality of the behaviour of proteins in sodium dodecyl sulphate/polyacryl-

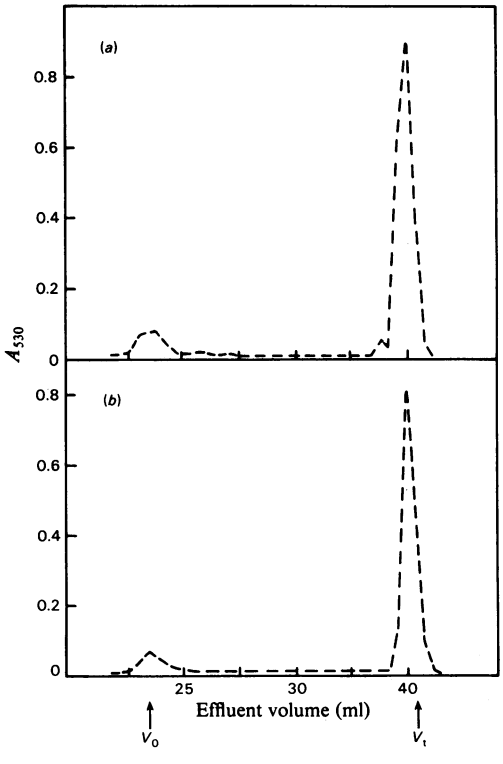

Fig. 9. *Bio-Gel P-30 chromatography of chondroitinase AC-II (a) and chondroitinase ABC (b) digests of the small proteoglycan*

Experimental details are given in the text. ----, A_{530} (carbazole reaction).

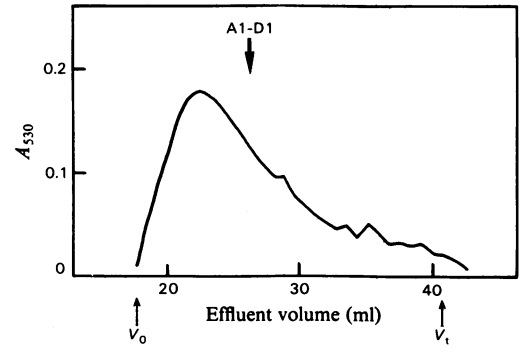

Fig. 10. *Sephadex G-200 chromatography of a papain digest of the small proteoglycan to determine size distribution of side chains*

Experimental details are given in the text. The elution position of chondroitin sulphate side chains of large cartilage proteoglycans (A1-D1 fraction) is indicated. ——, A_{530} (carbazole reaction).

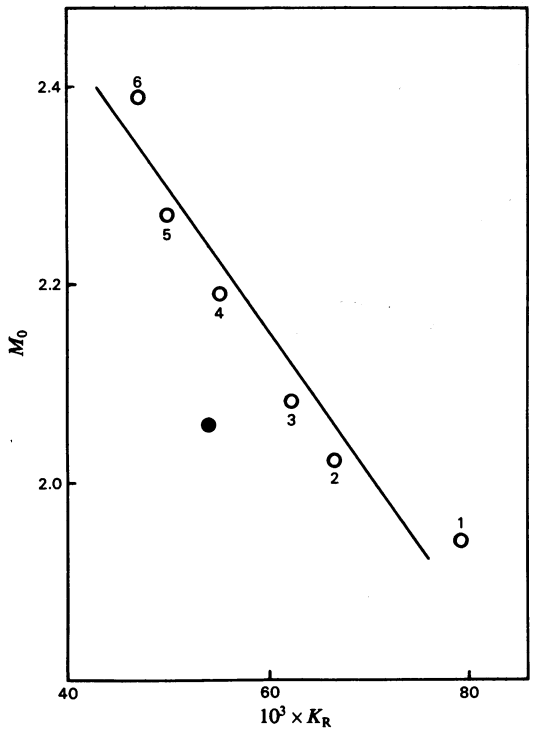

Fig. 11. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at various concentrations of polyacrylamide of chondroitinase-digested and reduced small proteoglycan*

Experimental details are given in the text. Plots of the free electrophoretic mobility versus retardation coefficient of reference proteins (O) and of the core protein of the small proteoglycan (●) are shown. Reference proteins were: phosphorylase *b* (1), bovine serum albumin (2), catalase subunit (3), ovalbumin (4), lactate dehydrogenase subunit (5) and carbonic anhydrase (6).

amide-gel electrophoresis. Such a plot indicated that the core protein had a lower apparent free electrophoretic mobility than should be expected from its retardation coefficient, when compared with the reference proteins (Fig. 11). Consequently, its behaviour on electrophoresis is non-ideal and would result in too high estimates of the molecular weight. The aberration could at least partly be due to the presence of oligosaccharides.

General discussion

The novel proteoglycan isolated is much smaller than and qualitatively different from previously described proteoglycans. It probably occurs in most types of cartilage. By using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without previous digestion with chondroitinase ABC it has also been identified in bovine tracheal cartilage and in different layers of bovine hip articular cartilage (results not shown). Since the latter cartilage does not contain blood vessels in its superficial layers, the small proteoglycan discussed cannot be derived from blood vessels. Furthermore, Kasarawa *et al.* (1979) and Vasan & Lash (1979) observed a slowly sedimenting proteoglycan in chondrocyte and tissue-culture experiments, most probably corresponding to the small proteoglycan. Swann *et al.* (1979) prepared a fraction from articular cartilage containing a major proportion of a small proteoglycan. This material had a molecular weight of 240000 and high contents of leucine, and probably represents a fraction enriched in the small proteoglycan discussed. Stanescu *et al.* (1977) identified and later Stanescu & Sweet (1981) isolated from baboon cartilage a low-buoyant-density proteoglycan with a high relative mobility on electrophoresis on composite agarose/polyacrylamide gels. This material had an amino acid composition similar to that of the small proteoglycan discussed in the present paper.

The function of the small proteoglycan is unclear. Undifferentiated mesenchyme, however, appears to contain a higher proportion of molecules of this kind (Kimata *et al.*, 1978; Vasan & Lash, 1979; Royal *et al.*, 1980). The amino acid composition is quite different from that of the large cartilage proteoglycans. The chondroitin sulphate chains are considerably larger. Therefore it is unlikely that the small proteoglycan represents a catabolic product of the large proteoglycans. The fact that it can be isolated under denaturing conditions indicated that it is not a product of degradation during isolation. It is probable that the small proteoglycan contains two chondroitin sulphate chains and a few oligosaccharides.

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