Biochem. J. (1984) **224**, 59–66
Printed in Great Britain

Characterization of proteoglycans from the calcified matrix of bovine bone

Ahnders FRANZÉN*† and Dick HEINEGÅRD*
**Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund, Sweden, and*
†Department of Stomatognathic Physiology, School of Dentistry, Carl Gustavs Väg 34, S-214 21 Malmö, Sweden

(*Received 24 April 1984/Accepted 7 August 1984*)

The proteoglycans characterized were those isolated from the calcified matrix of mature bovine bone [Franzén & Heinegård (1984) *Biochem. J.* **224**, 47–58]. The average molecular mass of the bone proteoglycan is 74600Da, determined by sedimentation-equilibrium centrifugation in 4M-guanidinium chloride. Its sedimentation ceofficient ($s^0_{20,w}$) is 3.04S. The apparent M_r of its core protein is 46000, estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the chondroitinase ABC-digested proteoglycan. A more likely molecular mass of the core protein is 30000Da, as calculated from the molecular mass and the protein content (40%) of the proteoglycan. The bone proteoglycan contains one or probably two chondroitin sulphate chains each with a molecular mass (weight-average) of 33700Da and several oligosaccharides both of the *N*-glycosidically and the *O*-glycosidically linked type. Antibodies against the homogeneous bone proteoglycans were raised in rabbits. An e.l.i.s.a. (enzyme-linked immunosorbent assay) method was developed that allowed specific quantification of bone proteoglycans at nanogram levels. The specificity of the antibodies was tested by using the e.l.i.s.a. method. The bone proteoglycan showed partial cross-reactivity with the small proteoglycan of cartilage. The antibodies were used to localize immunoreactivity of bone proteoglycans by indirect immunofluorescence in frozen sections of foetal bovine epiphysial growth plate. The fluorescence was entirely found in the primary spongiosa, and no fluorescence was found among the hypertrophied chondrocytes or in the region of provisional calcification.

Three populations of proteoglycans having different composition and additional glycosaminoglycan peptides have been isolated from the calcified matrix of mature bovine bone (Franzén & Heinegård, 1984). The compositions of the proteoglycans differ. Their glycosaminoglycan chains were of different size, indicating non-identity. The core protein of the major proteoglycan fraction had an apparent M_r of 46000 estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chondroitinase ABC digests.

Proteoglycans with core proteins showing similar molecular dimensions and with similar amino acid composition have also been identified in extracts of cartilage (Heinegård *et al.*, 1981), cervix (Uldbjerg *et al.*, 1983), cornea (Axelsson & Heinegård, 1975) and sclera (Cöster & Fransson, 1981). Such small proteoglycans have so far been identified in all connective tissues studied and may represent a ubiquitous proteoglycan type. It should be stressed, however, that the small proteoglycans differ, i.e. some such as those from sclera and tendon contain dermatan sulphate chains with high contents of iduronic acid, whereas those from cartilage contain chondroitin sulphate chains with no iduronic acid. The present study was initiated to characterize the major population of bone proteoglycans with respect to its structure and composition and to develop an immunoassay and to test some immunological relationships with the small proteoglycan of cartilage.

Abbreviation used: e.l.i.s.a., enzyme-linked immunosorbent assay.

Materials and methods

Materials

Molecular-mass calibration kit, alkaline phosphatase substrate (Naphthol AS-MX phosphate), chondroitinase AC II and chondroitinase ABC were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Superose CL-6B came from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-10 was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., and DE-52 anion-exchange DEAE-cellulose was bought from Whatman Chemicals, Maidstone, Kent, U.K.

The proteoglycans to be characterized represent the major component (HTP II) extracted from the calcified matrix of bone and purified as described in the preceding paper (Franzén & Heinegård, 1984).

Chemical methods

The glycosaminoglycans and the oligosaccharides were quantified separately. They were liberated from the proteoglycan by alkaline-borohydride treatment (5mg/ml) in 50mM-NaOH/1M-$NaBH_4$ for 48h at 45°C (Carlson, 1968). After neutralization by addition of 10M-acetic acid, the samples were freeze-dried, dissolved at 0.5mg/ml in distilled water and chromatographed on DEAE-cellulose (DE-52) columns as described elsewhere (Inerot & Heinegård, 1983). The oligosaccharides were eluted from the columns with several volumes of distilled water followed by 0.6M-pyridine/acetate buffer, pH6.0. The glycosaminoglycans were then eluted with 2M-HCl. Contents of hexosamines in the glycosaminoglycan and oligosaccharide fractions were determined with an automatic amino acid analyser after hydrolysis of samples in 4M-HCl (AristaR) for 10h at 100°C in tubes sealed under argon. The protein content (of proteoglycan dry weight) was calculated from the sum of the amino acids (see Franzén & Heinegård, 1984). Hexuronic acid contents of samples were determined by an automated version of the carbazole method (Heinegård, 1973). Sialic acid contents were determined by an automated version (Lohmander *et al.*, 1980) of the method described by Jourdain *et al.* (1971). Hydroxyproline was quantified by the method of Stegeman & Stalder (1967).

Analytical ultracentrifugation

The apparent molecular mass of the bone proteoglycan was determined by sedimentation-equilibrium centrifugation in 4M-guanidinium chloride by using the meniscus-depletion method described by Yphantis (1964). Bone proteoglycan was dissolved at 5mg/ml in 4M-guanidinium chloride (Ultrapure; Bethesda Research Laboratories, Bethesda, MD, U.S.A.) buffered with 5mM-Tris/HCl, pH7.0, and dialysed against 100vol. of the guanidinium chloride solution for 48h at 4°C. The sample was diluted to final concentrations of 0.15mg/ml, 0.30mg/ml, 0.45mg/ml and 0.60mg/ml by adding weighed portions of the dialysis residue. Centrifugation was performed in an MSE Centriscan analytical ultracentrifuge at 17000, 19000 and 21000rev./min at 20°C. A cushion of fluorocarbon oil (FC 70; 3M, Minneapolis, MN, U.S.A.) was included to permit optimal optical resolution also at the bottom of the cells. The distribution of the material was analysed both with the schlieren system and by monitoring the absorbance at 280nm. Sedimentation coefficients were determined in separate centrifugations at 55000rev./min of samples diluted to 0.55mg/ml, 1.20mg/ml, 1.80mg/ml and 2.40mg/ml. The sedimentation was followed by using the schlieren system, and scans were automatically taken at regular time intervals. The sedimentation coefficients were calculated from the formulae given in the MSE manual (Technical publication, supplement 73), and the apparent s^0 value was corrected to 20°C and water.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels with and without previous digestion with chondroitinase as described elsewhere (Franzén & Heinegård, 1984).

Molecular mass of the glycosaminoglycan chains

The glycosaminoglycans were liberated from the proteoglycan core protein by alkaline-borohydride treatment as described above and freeze-dried. The sample was dissolved in 0.25M-Na_2SO_4, pH7, and chromatographed on a Superose CL-6B column (0.6cm × 140cm), eluted with 0.25M-Na_2SO_4, pH7.0. The absorbance of the column effluent at 206nm was monitored with a Uvicord S photometer (LKB, Bromma, Sweden). M_r values were calculated as described by Wasteson (1971).

Characterization of the oligosaccharides and glycosaminoglycans

A sample of the purified bone proteoglycan (1.4mg) was treated with alkaline borohydride as described above under 'Chemical methods'. The freeze-dried material was dissolved in 1M-pyridine/acetate buffer, pH7.0, and chromatographed on a column (0.6cm × 140cm) of Bio-Gel P-10 eluted with 1M-pyridine/acetate buffer, pH7.0. After chromatography, the fractions were freeze-dried, dissolved in 0.5ml of distilled water and analysed for contents of hexose by the orcinol method (Kesler, 1967). The elution positions of hyaluronic

acid oligomers (prepared as described by Hascall & Heinegård, 1974) were used as a reference. Fractions representing each peak were pooled and freeze-dried. These pools were then dissolved in measured volumes of distilled water, and weighed samples were used for determination of contents of hexosamines (see under 'Chemical methods'). Other weighed samples were used for determination of the neutral sugar composition after hydrolysis of samples in 2M-trifluoroacetic acid for 3h at 100°C in sealed tubes. A Bio-Rad HPX-87P carbohydrate column was eluted with water. The effluent was monitored for contents of carbohydrate by using the orcinol reaction applied to a Technicon Autoanalyser (S. Lohmander, personal communication).

A sample (50μg) of the void-volume peak of the Bio-Gel P-10 chromatography, containing the glycosaminoglycans, was dissolved in 50μl of 0.1M-Tris/HCl buffer, pH8.0. Chondroitin sulphate was depolymerized by incubation with chondroitinase AC II (0.06 nominal unit/mg) for 5h at 37°C. Concentrated sodium acetate was then added to a final concentration of 0.5M, and the digest was chromatographed on a Sephadex G-50 column (0.5cm × 140cm) eluted at 4°C with 0.5M-sodium acetate, pH7.0. Fractions were monitored for content of hexuronic acid by using an automated version of the carbozole procedure (Heinegård, 1973).

Production of antibodies

The purified bone proteoglycan was used for raising antibodies in rabbits. Immunization was done subcutaneously in the neck with 1mg of bone proteoglycan in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Monthly booster injections of 0.5mg of the proteoglycan in Freund's incomplete adjuvant (Difco Laboratories) were given until a satisfactory titre was obtained. Antibodies directed against the bone proteoglycans were detected with an e.l.i.s.a. technique.

E.l.i.s.a.

Coating of poly(vinyl chloride) microtitre plates (Dynatech M29) was done overnight with 200μl/well of a solution (1μg/ml) of bone proteoglycan in 50mM-Na_2CO_3, pH9.6. The proteoglycans had been digested with chondroitinase ABC (0.01 unit/mg of proteoglycan) before use. The antigen solution to be assayed was preincubated overnight in incubation buffer, i.e. 0.15M-NaCl/5mM-sodium phosphate buffer, pH7.4 (PBS), containing 0.05% Tween 20, to which had been added an equal volume of antibody diluted at 1:2000 with incubation buffer. After coating, the wells were extensively rinsed with PBS/0.05% Tween 20 (PBS-Tw) to remove unbound proteoglycan. Samples (200μl) of preincubated antigen/antibody mixture were then pipetted into the well coated with bone proteoglycan, and available antibodies were allowed to react for 1h at 20°C. After extensive rinsing with PBS-Tw, 200μl of pig anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) diluted at 1:200 with incubation buffer and supplemented with 2mg of bovine serum albumin/ml were added to each well and allowed to bind to the rabbit antibodies bound to the wall of the well. After 4h at 20°C, the wells were rinsed with PBS-Tw, and 200μl of alkaline phosphatase substrate (Naphthol AS-MX phosphate) in 1M-diethanolamine/0.5mM-$MgCl_2$, pH9.8, was pipetted into each well. Incubation was performed at 20°C for 1h and the absorbance at 405nm was measured with a Titertec Multiscan Filterphotometer (Flow Laboratories, Rockville, MD, U.S.A.).

The specificity of the antibodies were tested. Powdered bovine diaphysis was extracted as described in the preceding paper (Franzén & Heinegård, 1984). The extract from the calcified bone matrix (guanidinium chloride/EDTA) was extensively dialysed at 4°C against 7M-urea/10mM-Tris/HCl/0.1M-sodium acetate buffer, pH6.0. The dialysis residue was chromatographed on a DEAE-cellulose (DE-52) ion-exchange column (11mm × 125mm) eluted with a sodium acetate gradient (0.1–1.2M) in 7M-urea, pH6.0. The column effluent was collected in fractions, which were analysed for contents of protein (A_{280}), hexuronic acid and sialic acid. Equal-sized samples of the fractions were diluted with the incubation buffer for e.l.i.s.a. and the immunoreactivity against bone proteoglycans was analysed by e.l.i.s.a.

Immunofluorescence

Calf femoral epiphyses (age 4 months *in utero*) were carefully dissected free within 1h after the death of the mother cow. Tissue specimens (about 2mm × 2mm × 4mm) were embedded in CM-cellulose (Tissue-Tek II; Miles Laboratories, Kankakee, IL, U.S.A.), frozen on blocks of solid CO_2 on a cryostat stage and sectioned at −30°C into 8μm sections. Each section was picked up on slides coated with gelatin. After being dried in the cryostat for about 1h, the sections were fixed in absolute ethanol for 5min and then left to dry at room temperature. Each section was incubated at 37°C for 30min with 50μl of anti-(bone proteoglycan) antibodies diluted 1:10 with PBS. Control sections were similarly incubated with diluted (1:10) pre-immune serum from the rabbit. The sections were extensively washed with PBS for 1h

at 20°C. Pig anti-(rabbit IgG) antibodies conjugated with fluorescein isothiocyanate (Dakopatts, Copenhagen, Denmark) and diluted to 1:25 with the buffer (50 μl) were added to each of the sections. After incubation at 37°C for 30 min, the sections were carefully washed at 20°C for 4 h with the buffer, then distilled water, and dried. Distribution of fluorescence was studied by using a Leitz microscope and photographed at 20× magnification with Kodak Ektachrome (400 ASA) film.

Results and discussion

Composition analysis

The bone proteoglycan has a high protein content of almost 40%, comparable with that of small proteoglycans from other sources (for references see Heinegård & Paulsson, 1984). Other similarities with such proteoglycans are relatively low contents of uronic acid and hexosamines. The amino acid composition is typical for small proteoglycans, showing high contents of aspartic acid/asparagine, glutamic acid/glutamine and leucine (Franzén & Heinegård, 1984). No hydroxyproline could be demonstrated. The glucosamine/galactosamine ratio of the proteoglycan was 0.15. The presence of mannose, glucosamine and sialic acid can be attributed to oligosaccharides. Interestingly, the small proteoglycan isolated from cartilage contains no sialic acid (Heinegård *et al.*, 1981), showing that the molecules are not identical.

Characteristics of the carbohydrate side chains

Oligosaccharides and glycosaminoglycans were liberated by treatment of the proteoglycan with alkaline borohydride. Chromatography on Bio-Gel P-10 (Fig. 1) revealed one major orcinol-reactive component that was eluted in the void volume, which represents glycosaminoglycan chains. The oligosaccharides distributed in several components corresponding to the elution position of four to 14 hyaluronic acid monosaccharide units; five (I–V) fractions were pooled as indicated (Fig. 1).

A sample of the glycosaminoglycans in the void-volume fraction (I) was digested with chondroitinase AC II and chromatographed on Sephadex G-50. This chromatogram showed that the enzyme completely depolymerized the glycosaminoglycans to disaccharides, showing that the side chains contain only chondroitin sulphate (results not shown).

The compositions of the four oligosaccharide fractions (II–V) isolated after alkaline-borohydride treatment were determined (Table 1). All fractions contained some galactosamine, possibly due to a contamination with low-molecular-mass chondroitin sulphate. The fractions (II and III) representing the longest oligosaccharides did not contain any hexosaminitol, indicating the presence in these fractions of only oligosaccharides that are *N*-glycosidically linked to the protein core and therefore stable to the alkaline-borohydride treatment. These two oligosaccharide fractions differed with respect to composition. The fractions that

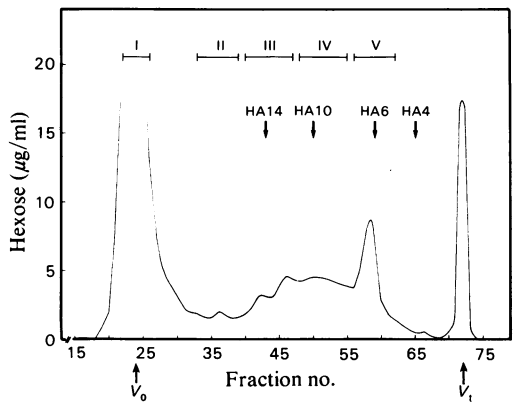

Fig. 1. *Gel chromatography of oligosaccharides obtained from bone proteoglycan*

Alkaline-borohydride-treated bone proteoglycan was subjected to Bio-Gel P-10 chromatography (eluent 1 M-pyridine/acetate buffer, pH 7.0). ——, A_{420} (orcinol reaction). Elution positions for hyaluronic acid (HA) oligomers are shown as reference. V_0, Void volume; V_t, total volume. Further information is given in the text.

Table 1. *Composition of the oligosaccharide fractions II–V prepared by chromatography on Bio-Gel P-10 of alkaline-borohydride-treated bone proteoglycans*

For experimental details see the text.

	Composition [μg/mg dry wt. (residues/residue of glucosamine)]				
	Galactosaminitol	Glucosamine	Galactosamine	Galactose	Mannose
II	—	0.89 (1)	2.47 (2.78)	1.03 (1.16)	0.98 (1.10)
III	—	2.38 (1)	1.82 (0.76)	1.26 (0.52)	2.81 (1.17)
IV	0.357 (0.18)	2.04 (1)	1.39 (0.68)	0.86 (0.42)	3.76 (1.85)
V	0.408 (1.41)	0.29 (1)	0.32 (1.11)	0.68 (2.35)	1.95 (6.75)

were eluted retarded (IV and V), and therefore represented smaller oligosaccharides, contained galactosaminitol as well as glucosamine and hexose. Since these oligosaccharides contained galactosaminitol, it is likely that they were *O*-glycosidically linked to the proteoglycan core protein. The sialic acid contents of the four fractions were quite similar. At present, we cannot explain the high content of mannose in the two retarded fractions (IV and V). Mannose would not normally be found in *O*-glycosidically linked oligosaccharides. The data, however, show that the bone proteoglycan contains chondroitin sulphate chains as well as both *N*- and *O*-glycosidically linked oligosaccharides.

Molecular mass and sedimentation coefficient

The molecular mass of the bone proteoglycan is 74600Da as determined by sedimentation-equilibrium centrifugation in 4M-guanidinium chloride (Fig. 2*a*). The same value was obtained

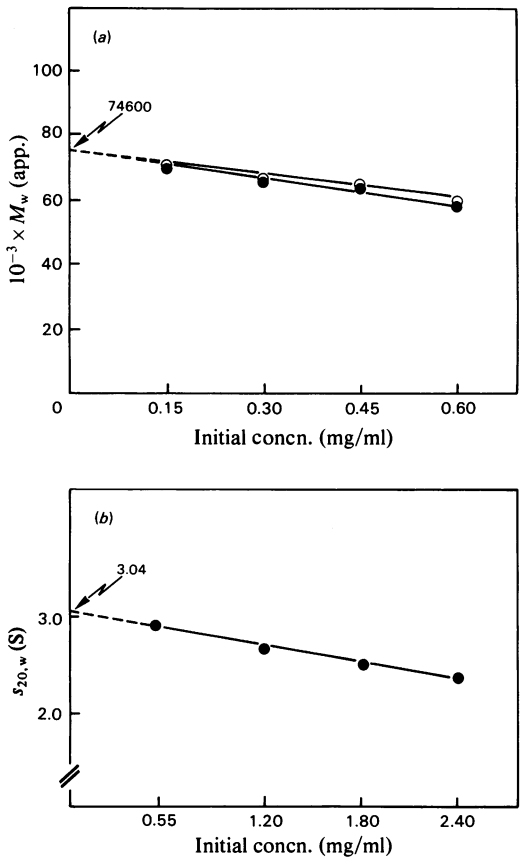

Fig. 2. (*a*) *Sedimentation characteristics of the bone proteoglycan and* (*b*) *determination of* $s^0_{20,w}$ *of the bone proteoglycan*

(*a*) Molecular mass of the bone proteoglycan was determined by sedimentation-equilibrium centrifugation in 4M-guanidinium chloride. The distribution of material was monitored by using absorbance at 280nm (○) and with schlieren optics (●). For further information see the Materials and methods section. The sedimentation coefficients were calculated, corrected to 20°C and water and extrapolated to zero concentration.

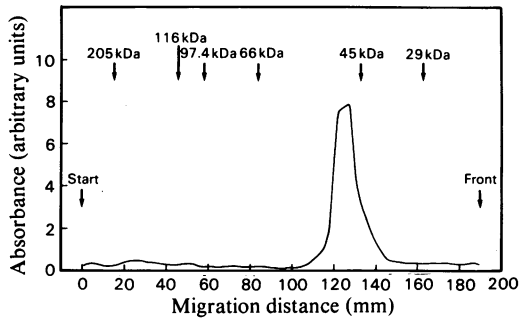

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the bone proteoglycan after chondroitinase ABC digestion and reduction*

The gel was scanned by using a soft-laser scanner. The apparent M_r of the proteoglycan core protein was determined by comparing the migration distance with those of several reference proteins. Experimental details are given in the text.

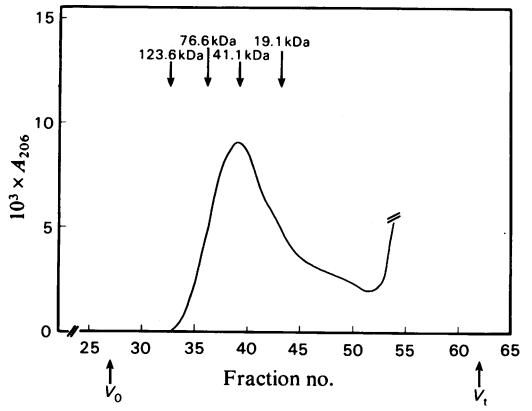

Fig. 4. *Determination of the apparent molecular mass of the glycosaminoglycan chains*

The bone proteoglycan was treated with alkaline borohydride and the liberated chains were chromatographed on Sepharose 6B (eluent 0.25M-Na_2SO_4, pH7.0). The column was calibrated with chondroitin sulphate chains of known molecular mass. The column effluent was continuously monitored for absorbance at 206nm with a Uvicord Filterphotometer. Experimental details are given in the Materials and Methods section. V_0, Void volume; V_t, total volume.

both with measurements involving the use of schlieren optics and with measurements of the absorbance at 280nm, indicating that the contribution of free chondroitin sulphate chains, if any, was small. Determinations at three different centrifugation speeds (17000, 19000 and 21000rev./min) gave identical values, indicating that the proteoglycan preparation was less polydisperse than often seen. Because of the great similarities in composition between the cartilage small proteoglycan and the bone proteoglycan, the partial specific volume, i.e. 0.59ml/g, obtained for the cartilage proteoglycan was used for calculating the molecular mass. Interestingly, the molecular-mass values were almost identical for the two proteoglycans. The $s^{\circ}_{20,w}$ value for the bone proteoglycan was 3.04S, determined by sedimentation-velocity centrifugation at 55000rev./min in 4M-guanidinium chloride (Fig. 2*b*). The cartilage small proteoglycan had a similar $s^{\circ}_{20,w}$ value of 3.11S, determined under identical conditions (Heinegård *et al.*, 1981).

Apparent M_r of the proteoglycan core protein

The core protein prepared by digestion of the bone proteoglycan with chondroitinase ABC has an apparent M_r of 46000, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 8%-polyacrylamide gels (Fig. 3). This value represents an overestimate, owing to the shortcomings of the technique used. A better estimate of the molecular mass of the core protein is 30000Da, calculated from the protein content and the molecular mass of the intact proteoglycan. The electrophoresis, however, shows that the core protein is monodisperse, i.e. one well-defined component. Interestingly, proteoglycans from other sources, such as cartilage (Heinegård *et al.*, 1981), sclera (Cöster & Fransson, 1981), cervix (Uldbjerg *et al.*, 1983), tendon (K. Vogel & D. Heinegård,

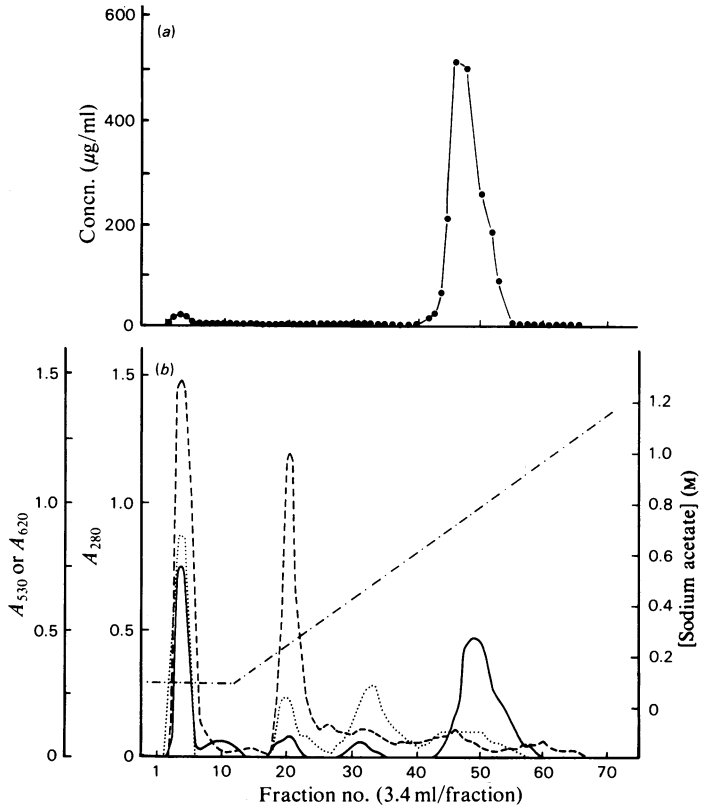

Fig. 5. *Specificity of e.l.i.s.a. of bone proteoglycan*

An extract with guanidinium chloride/EDTA of the calcified matrix of bovine bone was subjected to DEAE-cellulose (DE-52) chromatography (eluent 7M-urea, pH6.0). The column was eluted with a gradient of sodium acetate (·–·–·). (*a*) Immunoreactivity against bone proteoglycans measured as inhibition in e.l.i.s.a. (*b*) Protein (A_{280}, ----), uronic acid by the carbozole reaction (A_{530}, ——) and sialic acid (A_{620}, ·····). Experimental details are given in the Materials and methods section.

unpublished work) and aorta (S. Gardell & D. Heinegård, unpublished work), have similar size, and the core protein migrates on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 44000–46000. It is possible that these proteoglycans represent a group of closely related molecules.

Molecular mass of the glycosaminoglycan chains

The molecular masses of the glycosaminoglycan side chains were determined by gel chromatography on Superose 6B of alkaline-borohydride-treated bone proteoglycans (Fig. 4). Number-average and weight-average molecular masses were calculated in accordance with Wasteson (1971) and found to be to 18600 and 33700 Da respectively. These values are similar to those obtained with the other similar proteoglycans discussed above. The average size of the glycosaminoglycan chains from this type of proteoglycan, however, is almost twice that of the large proteoglycans from bovine nasal cartilage (Heinegård *et al.*, 1981).

Characteristics of the proteoglycan

From the size determination of the intact bone proteoglycan and of fragments thereof, it is possible to postulate a tentative model of the molecule. The molecule appears to contain a core protein with an estimated molecular mass of 30000 Da and one or two large glycosaminoglycan side chains of about 34000–35000 Da. The molecule in addition contains both *N*-linked and *O*-linked oligosaccharides.

Specifity of the antibodies

The purified bone proteoglycan was used for raising antibodies in rabbits. The specificity of the antibodies was tested with an inhibition e.l.i.s.a. The macromolecules bound in the calcified matrix of bovine bone were extracted as described in the preceding paper (Franzén & Heinegård, 1984). The extract was chromatographed on a DEAE-cellulose ion-exchange column eluted with a sodium acetate gradient, and the effluent fractions were analysed for contents of protein, hexuronic acid and sialic acid (Fig. 5*b*). Three main polyanionic components were bound to the column and were eluted well separated from each other at different concentrations of sodium acetate. The second component was identified as a bone sialoprotein, probably corresponding to that originally identified by Andrews *et al.* (1967). The bone proteoglycans, identified by their high hexuronic acid content, were recovered in the third main peak. The immunoreactivity against bone proteoglycans was determined by measuring the inhibition of each fraction in the e.l.i.s.a. (Fig. 5*a*). More than 98% of the bone proteoglycan immunoreactivity was recovered in the expected late-eluted peak, showing the specificity of the assay. A very small portion (less than 2%) was found in the fractions containing material not bound to the column, probably representing a small proportion of bone proteoglycan not bound to the column. The data show that the antibodies did not cross-react (measured as inhibition in the e.l.i.s.a.) with any other component in the extract of bone.

Cross-reactivity of small proteoglycans

The small proteoglycans of bone and cartilage showed similarities in the amino acid composition as well as in their hexosamine and hexuronic acid contents. Furthermore, the molecular masses of the two proteoglycans, as well as the dimensions of the core proteins, were quite similar. Therefore the immunological relationship between the two molecules was studied (Fig. 6). The wells in the microtitre plate were coated with chondroitinase ABC-digested bone proteoglycan. The inhibition in an assay for bone proteoglycan was tested with intact as well as with chondroitinase ABC-digested small proteoglycans from bone and cartilage. All preparations reacted with the antibodies. Surprisingly, the undigested proteoglycans showed a higher degree of inhibition than did those that had been digested (Fig. 6). The small cartilage proteoglycan was a less efficient inhibitor than the bone proteo-

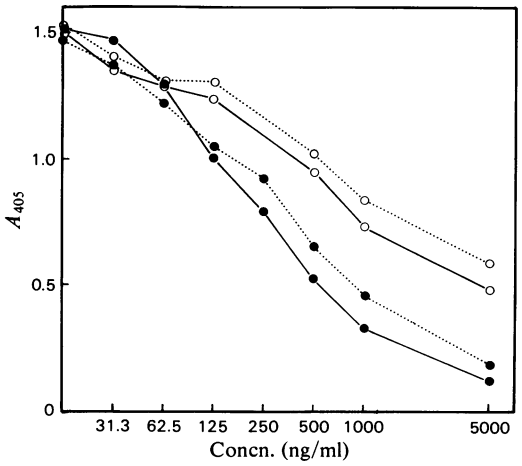

Fig. 6. *Immunological cross-reactivity of bone proteoglycan and cartilage small proteoglycan*

The results of e.l.i.s.a. for bone proteoglycans and inhibition with the fractions indicated are shown. ●—●, Bone proteoglycan; ●·····●, bone proteoglycan digested with chondroitinase ABC; ○—○, cartilage small proteoglycan; ○·····○, cartilage small proteoglycan digested with chondroitinase ABC.

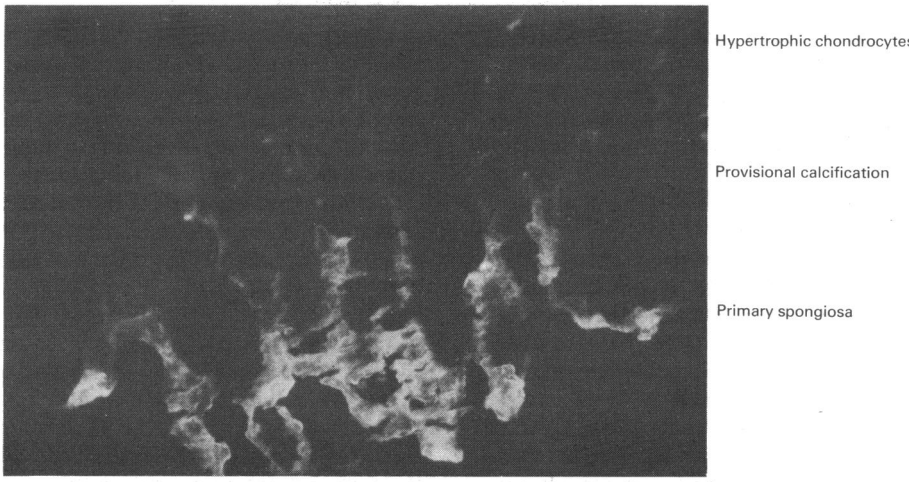

Fig. 7. *Localization of bone proteoglycan by immunofluorescence*
Frozen unfixed sections of foetal-calf epiphysis were stained for bone proteoglycans by using double-antibody immunofluorescence. Magnification ×90.

glycan, indicating only partial cross-reactivity between the two small proteoglycans.

Immunofluorescence

The antibodies directed against the bone proteoglycans were used to localize these molecules within unfixed cryostat sections of foetal-calf epiphysis. Indirect immunofluorescence with a double-antibody technique, including anti-(bone proteoglycan) antibodies and anti-(rabbit IgG) antibodies labelled with fluorescein isothiocyanate was used. Strong immunofluorescence with the specific antibody was only found in the primary spongiosa, whereas no immunofluorescence was detected among the hyperthrophic chondrocytes or in the early stages of the provisional calcification (Fig. 7). The normal cartilage of the resting zone showed weak immunofluorescence, probably a result of some cross-reactivity between the small proteoglycans from cartilage and from bone. Substituting the specific antiserum with a pre-immunization serum abolished all immunofluorescence (results not shown).

Grants were obtained from the Swedish Medical Research Council (05668), Folksams Yrkesskadors Stiftelse, Kock's Stiftelser, Österlunds Stiftelse, Konung Gustaf V:s 80-årsfond and the Faculties of Medicine and Odontology, University of Lund.

References

Andrews, A. T. de B., Herring, G. M. & Kent, P. W. (1967) *Biochem. J.* **104**, 705–715
Axelsson, I. & Heinegård, D. (1975) *Biochem. J.* **169**, 517–530
Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
Cöster, L. & Fransson, L.-Å. (1981) *Biochem. J.* **193**, 143–153
Franzén, A. & Heinegård, D. (1984) *Biochem. J.* **224**, 47–58
Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4242–4249
Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201
Heinegård, D. & Paulsson, M. (1984) in *Connective Tissue Biochemistry* (Piez, K. & Reddi, H., eds.), pp. 277–328, Elsevier/North-Holland, New York
Heinegård, D., Paulsson, M., Inerot, S. & Carlström, C. (1981) *Biochem. J.* **197**, 355–366
Inerot, S. & Heinegård, D. (1983) *Collagen Relat. Res. Clin. Exp.* **3**, 245–262
Jourdain, G. W., Dean, L. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 430–435
Kesler, R. B. (1967) *Anal. Chem.* **39**, 1416–1422
Lohmander, S., DeLuca, S., Nilsson, B., Hascall, V. C., Caputo, C., Kimura, J. & Heinegård, D. (1980) *J. Biol. Chem.* **255**, 6084–6091
Neville, D. (1971) *J. Biol. Chem.* **246**, 6328–6334
Stegeman, H. & Stalder, K. (1967) *Clin. Chim. Acta* **18**, 267–273
Uldbjerg, N., Malmström, A., Ekman, G., Sheehan, J., Ulmsten, U. & Wingerup, L. (1983) *Biochem. J.* **209**, 497–503
Wasteson, Å. (1971) *J. Chromatogr.* **59**, 87–97
Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317