Biochem. J. (1986) 240, 41–48 (Printed in Great Britain)

Electron-microscopic and electrophoretic studies of bovine femoral-head cartilage proteoglycan fractions

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Proteoglycans (A1D1) extracted from bovine femoral-head cartilage were examined by electron microscopy using benzyldimethylammonium chloride as a spreading agent. The preparation contained a mixture of particles, some with a 'beaded' structure and a contiguous filamentous 'tail' at one end and others which appeared as round 'blobs', some of which also had filamentous tails. Previous electron-microscopic studies of proteoglycan monomers have indicated that their length distributions were apparently unimodal, a finding that contrasted with agarose/polyacrylamide-gel-electrophoresis results, which generally indicated two bands. In the present study proteoglycans isolated from the slowly migrating electrophoretic band were shown to be predominantly the larger molecules of beaded appearance, whereas the rapidly migrating proteoglycans were predominantly molecules with the 'blob-like' appearance. Gel-filtration, isopycnic-density-gradient-centrifugation and rate-zonal-centrifugation techniques were evaluated as means of proteoglycan fractionation by electron microscopy and agarose-gel electrophoresis. Rate-zonal centrifugation in mixed-salt gradients of caesium chloride/4 M-guanidinium chloride yielded the most effective fractionation.

INTRODUCTION

The mechanical resilience and water-retention properties of cartilage are maintained by the presence of large polyanionic proteoglycans. These molecules occur mainly as high-M_r proteoglycan aggregates formed as a result of the specific interaction of proteoglycan monomers and hyaluronate (Hardingham & Muir, 1972). This interaction is further stabilized by link-protein molecules (Hardingham, 1979).

The physico-chemical studies on which knowledge of proteoglycans is based have mostly been performed on the high-buoyant-density molecules purified from bovine nasal and pig laryngeal cartilage (Kitchen & Cleland, 1978; Sheehan *et al.*, 1978). These proteoglycans have a range of M_r values from 10^6 to 3×10^6 and an M_w of 2.3×10^6. Studies on articular-cartilage high-buoyant-density proteoglycans from bovine femoral-head (Lyon *et al.*, 1983), bovine condylar (Swann *et al.*, 1979) and chick-limb-bud cartilage (Shogren *et al.*, 1982) indicate that these proteoglycans are smaller [M_w values 1.16×10^6, 1.3×10^6 and $(1.05-1.96) \times 10^6$ respectively].

Electron-microscopic studies using Kleinschmidt spreading techniques have provided support for the proposed models of both the proteoglycan monomer and aggregate (Buckwalter & Rosenberg, 1982; Heinegård *et al.*, 1978; Kimura *et al.*, 1978; Rosenberg *et al.*, 1970, 1975). The length distribution of the proteoglycan monomers is apparently unimodal, with a range of lengths between 20 and 450 nm.

The unimodal length distribution for the proteoglycans observed in the aforementioned electron-microscopic studies contrasts with the generally bimodal distributions indicated from agarose/polyacrylamide-gel-electrophoresis data (Heinegård *et al.*, 1985*a*,*b*; Roughley & Mason, 1976; Stanescu & Stanescu, 1983). However, the mechanism by which electrophoretic separation of proteoglycans is achieved is not fully understood. Recently, though, Heinegård *et al.* (1985*a*) demonstrated a difference in migration distance, on agarose/polyacrylamide gels, for chondroitin sulphate-rich and keratan sulphate-rich aggregating proteoglycans prepared from bovine nasal cartilage. The larger, chondroitin sulphate-rich, proteoglycan (M_w 3×10^6) showed a slower migration than the smaller, keratan sulphate-rich, proteoglycan (M_w 1.3×10^6).

In the present study both the discrepancy between the electron-microscopically and electrophoretically observed distributions of proteoglycans and an effective means of fractionating large and small proteoglycans are addressed.

MATERIALS AND METHODS

Preparation of proteoglycan

High-buoyant-density proteoglycans (A1D1, $\rho \geqslant 1.58$ g/ml) from bovine femoral-head cartilage (15–18-month-old animals), obtained fresh from the abattoir, were prepared essentially as described by Lyon *et al.* (1983), but the proteinase-inhibitor mixture also contained 0.01 M-*N*-ethylmaleimide.

Preparation of [^{14}C]- and [^{3}H]-proteoglycan

Proteoglycan aggregates (A1 fraction) from bovine femoral-head cartilage (15–18-month-old animals), were prepared essentially as described by Lyon *et al.* (1983), but the proteinase-inhibitor mixture also contained 0.01 M-*N*-ethylmaleimide. The aggregates were extensively dialysed against 5 mM-sodium phosphate, pH 7.4, containing 0.01 M-EDTA at 6 °C. The non-diffusable

Abbreviations used: M_w, weight-average relative molecular mass; BAC, benzyldimethylammonium chloride.

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material (1.7 mg of uronate/ml) was divided; one portion (64 ml) was labelled with 250 μCi of [^{14}C]acetic anhydride and the other portion (94 ml) was labelled with 25 mCi of [^{3}H]acetic anhydride. After stirring for 1 h, each solution was extensively dialysed against 5 mM-sodium phosphate, pH 7.4, containing inhibitors and then against 4 M-guanidinium chloride, pH 7.4. The density of these solutions was adjusted to 1.5 g/ml by the addition of solid CsCl. 'Dissociative' density-gradient centrifugation was performed in an 8 × 50 ml A1 angle-head rotor in an MSE Prepspin 50 centrifuge at 87000 $g_{av.}$ for 70 h at 15 °C. Tubes were unloaded from the bottom via a capillary tube, and the proteoglycan-containing fractions (A1D1), with $\rho \geqslant 1.58$ g/ml, were pooled. This yielded [^{14}C]- and [^{3}H]-proteoglycan preparations with specific radioactivities of 23 nCi/mg (i.e. approx. 1.6 acetyl groups/mol) and 900 nCi/mg (i.e. approx. 2.5 acetyl groups/mol) respectively.

The density of the A1D1 fraction, containing ^{14}C-labelled proteoglycans, was adjusted to 1.61 g/ml with solid CsCl and it was subjected to a second density-gradient centrifugation. Tubes were unloaded (as above) into 11 equal fractions and fractions 1–6 were analysed further by agarose-gel electrophoresis and electron microscopy.

Agarose-gel electrophoresis

Proteoglycans (< 0.5 mg/ml) were electrophoresed in horizontal 1%-(w/v)-agarose slab gels (14 wells, 11 cm × 14 cm, 4 mm thickness) in a horizontal system for submerged-gel electrophoresis (model H5; BRL, Paisley, Renfrewshire, Scotland, U.K.). The gel (50 ml) and electrophoresis buffer (850 ml) was 40 mM-Tris/acetate/1 mM-sodium sulphate/0.1% SDS, pH 6.8. Samples were prepared as described by Heinegård *et al.* (1985*b*) and electrophoresis was performed for 2.5 h at a constant current of 75 mA (30–35 V). Gels were fixed in methanol/acetic acid/water (50:7:43, by vol.), stained with 0.2% (w/v) Toluidine Blue in 3% (v/v) acetic acid and destained in 3% (v/v) acetic acid (Heinegård *et al.*, 1985*b*). For some electron-microscopy experiments proteoglycans were removed from the agarose gel after electrophoresis. Proteoglycan (A1D1) was electrophoresed as above and the gel was cut in two. One half was fixed, stained and destained to reveal the migration position of the proteoglycans. After realigning the two halves of the gel the regions corresponding to the slow and fast bands were sliced from the unstained portion. The two gel strips, and one taken from a region where no proteoglycans were present, were placed in separate dialysis bags filled with electrophoresis buffer (1 ml). Electrophoresis was then performed as described above, but in the absence of SDS in the electrophoresis buffer. The polarity was reversed for the final 3 min of the run to detach proteoglycans from the surface of the dialysis bag. The solutions were removed from the dialysis bags for electron microscopy.

Analytical methods

Uronate content of fractions from density-gradient centrifugation and gel chromatography was determined by an automated carbazole assay (Heinegård, 1973) based on the modified procedure of Bitter & Muir (1962), with glucuronolactone as standard. Radioactivity was measured in a Packard Tri-Carb 300 liquid-scintillation counter by the method of Nieduszynski *et al.* (1980).

Column chromatography

Proteoglycans (10 ml, 2 mg/ml) were chromatographed on a column of Sepharose CL-2B (155 cm × 1.8 cm) in 4 M-guanidinium chloride, pH 7.4 (flow rate 14 ml/h). The column effluent was monitored for uronate and fractions (5.2 ml) were pooled as shown (Fig. 4*a* below). The column was calibrated with glucuronolactone and Blue Dextran.

Rate-zonal centrifugation

(*a*) Analytical. [^{3}H]Proteoglycans (100 μl, 1 mg/ml) in 4 M-guanidinium chloride, pH 7.4, were layered on to linear gradients (5 ml) formed from 4 M-guanidinium chloride/CsCl [density (ρ) 1.1–1.4 g/ml]. Centrifugation was performed in a MSE Prepspin 50 centrifuge at 180000 $g_{av.}$ and 20 °C for 3.5 h in a swing-out rotor (6 × 5.5 ml, A1). Tubes were unloaded from the meniscus and fractions (270 μl) were counted for ^{3}H radioactivity. Fractions were pooled as shown (Fig. 6*a* below).

(*b*) Preparative. [^{3}H]Proteoglycans (1 ml, 3 mg/ml) in 4 M-guanidinium chloride, pH 7.4, were layered on to linear gradients (24 ml) formed from 4 M-guanidinium chloride/CsCl (ρ 1.15–1.45 g/ml). Centrifugation was performed in a MSE Prepspin 50 centrifuge at 70000 $g_{av.}$ and 20 °C for 16 h in an angle rotor (8 × 50 ml, A1). Tubes were unloaded from the bottom via a capillary tube and fractions (1.2 ml) were counted for ^{3}H radioactivity. Fractions were pooled as shown in Fig. 6(*b*) (below).

Electron microscopy

Carbon films (approx. 2 nm thick) were made by evaporation of carbon on to freshly cleaved mica. The films were floated off on water and deposited on 600- or 400-mesh grids (Gilder Grids Ltd., Grantham, Lincs., U.K.). Spreading methods used in DNA research (Koller *et al.*, 1969; Lang & Mitani, 1970) were adapted for the present study. Solutions of proteoglycans (0.05–0.2 μg/ml) in 50 mM-magnesium acetate were put into a Teflon or plastic Petri dish. Graphite powder was sprinkled on the surface, which was then touched with the tip of a plastic pipette containing a solution (1 g/100 ml) of BAC in water so that approx. 1 μl was spread on the surface, displacing the graphite to the edge of the dish. After 5–15 min, carbon-coated grids were touched to the surface, stained for 1–2 s in a solution of uranyl acetate [1 mM in 95% (v/v) ethanol] and finally washed for 1–2 s in 95% (v/v) ethanol. After drying in air the grids were usually subjected to either rotary or unidirectional shadowing at 7–12° with platinum/carbon. Electron microscopy was performed with a JEOL 100CX scanning transmission instrument at 80keV. Contour length measurements were made by tracing over photographic enlargements placed on a digital drawing pad (British Micro, Watford, Herts., U.K.) connected to a BBC microcomputer.

RESULTS

Electron microscopy and agarose-gel electrophoresis

An electron micrograph of bovine femoral-head proteoglycans (A1D1), spread using BAC, is shown in Fig. 1(*a*). The preparation was stained and very lightly shadowed with platinum/carbon and shows a mixture of

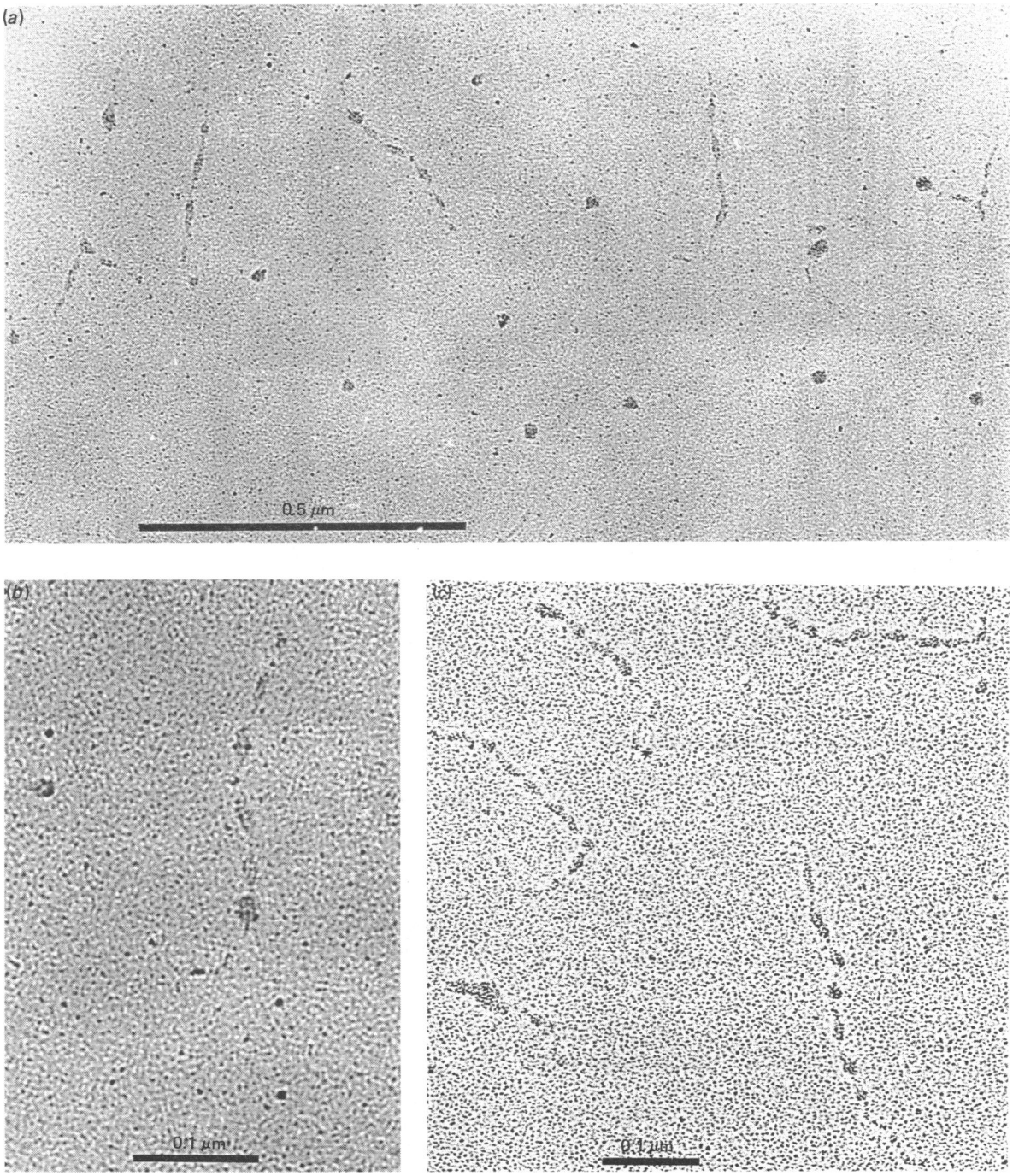

Fig. 1. Electron micrographs of proteoglycans spread in BAC monolayers

(*a*) Proteoglycans spread on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate (for experimental details, see the text). The molecules appear as flexible 'beaded' chains of various lengths and as round 'blobs', both of which have in many cases a fine filamentous tail. (*b*) A proteoglycan monomer under the same conditions as those described above, highlighting the beaded appearance and the filamentous tail. The outline of the protein core can be seen by a negative-staining contrast effect of the collapsed chondroitin sulphate chains. (*c*) Proteoglycans revealed by rotary shadowing with platinum/carbon at an angle of 15°, indicating that the beaded appearance is not an artefact due to the uranyl acetate alone.

particles, some with a pronounced 'beaded' structure with a contiguous filamentous 'tail' at one end, and others appear as round 'blobs', some of which also carry a filamentous tail. The beaded effect on the larger molecules is reproducible and possibly represents a clustering of the chondroitin sulphate chains which are collapsed around the protein core. At higher magnifications a positive staining effect of the protein core running through the collapsed chondroitin sulphate domain may be discerned (Fig. 1*b*). More extensive rotary shadowing

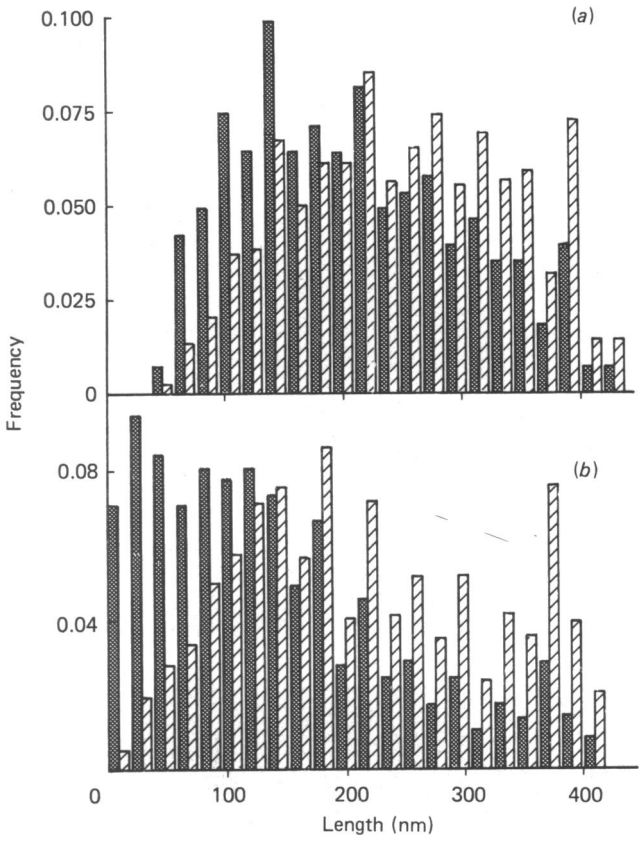

Fig. 2. Histograms of proteoglycan length distributions

(*a*) Proteoglycan length distributions excluding the blobs. The number frequency $[f(N_i) = N_i/\Sigma_i N_i]$ and weight frequency $[f(N_i \cdot L_i) = N_i L_i/\Sigma_i N_i L_i]$ are shown as stippled and hatched bars respectively. (*b*) Proteoglycan length distributions including the blobs. Stippled and hatched bars defined as in (*a*). The diameter of the blobs, plus the length of an attached filamentous tail, were used as a measure of the length.

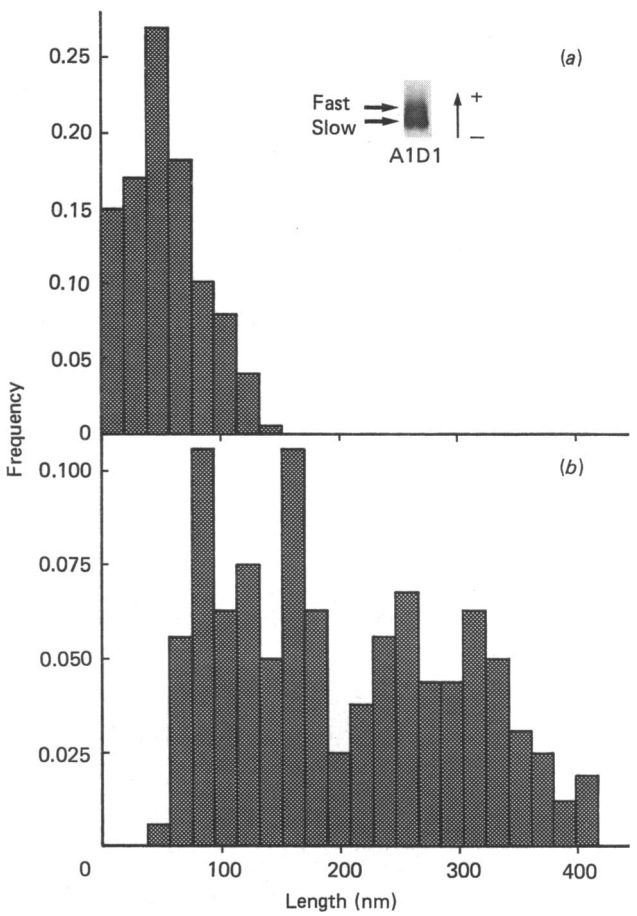

Fig. 3. Histograms of the length distributions of proteoglycans isolated from the two electrophoretic bands observed on 1% (w/v) agarose gels

The length distribution frequencies $[f(N_i) = N_i/\Sigma_i N_i)]$ of the proteoglycans isolated from (*a*) fast- and (*b*) slow-migrating bands (see the inset). For experimental details, see the text.

(Fig. 1*c*) can be used to enhance the contrast of the beaded structure, though there is a danger under these conditions of obscuring the finer structure.

A number-average length for the distribution is given by:

$$L_n = \Sigma_i N_i \cdot L_i / \Sigma_i N_i$$

and a weight-average length may be defined as:

$$L_w = \Sigma_i N_i \cdot L_i^2 / \Sigma_i N_i \cdot L_i$$

where N_i is the number of molecules with length L_i. Histograms of the length and weight frequency distributions of particles, excluding the blobs, are shown in Fig. 2(*a*) and yield a number-average and a weight-average of 214 nm and 255 nm respectively. Though the expression of a weight average for the proteoglycans by this method is only an approximation in that it assumes a common mass per unit length, it is useful, since all other methods employed in the analysis yield a weight-average view as compared with the number-average obtained by electron microscopy. The distributions when the blobs are recorded as proteoglycans are shown in Fig. 2(*b*). The statistics yield a number-average and a weight-average of 143 nm and 218 nm respectively.

Upon electrophoresis on agarose gels a distinct bimodal distribution in the staining pattern of the proteoglycans is observed. The number-frequency distributions of the particles obtained from each band (Figs. 3*a* and 3*b*) shows that the faster-moving band is primarily composed of small proteoglycans that are here revealed as blobs and short rods.

The electron-microscopic and electrophoretic techniques have been used to assess the effectiveness of column chromatography, density-gradient and rate-zonal centrifugation as means of proteoglycan fractionation.

Column chromatography

Proteoglycans were fractionated on Sepharose CL-2B (Fig. 4*a*) and were eluted as a single peak with $K_{av.}$ 0.4. The peak was divided into three fractions, and portions were analysed by agarose-gel electrophoresis (Fig. 4*a*) and electron microscopy (Fig. 4*b*). Fraction A shows only one band on electrophoresis and few blobs can be seen on electron micrographs (Fig. 4*b*,i). Fractions B and C both exhibit two bands on electrophoresis, and a

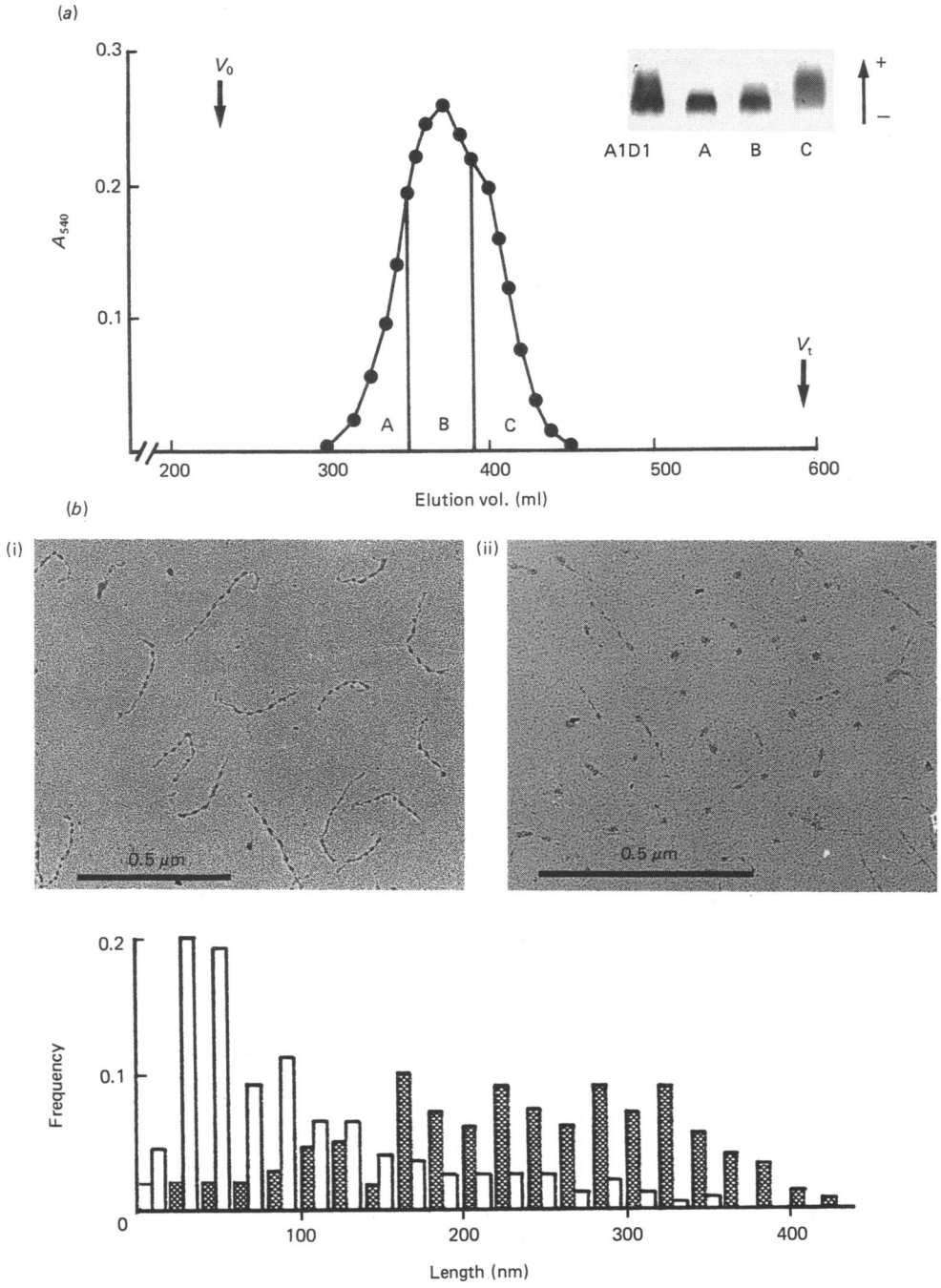

Fig. 4. Proteoglycan fractionation by gel filtration

(a) Proteoglycan (A1D1) was chromatographed on Sepharose CL-2B in 4 M-guanidinium chloride, pH 7.4 (see the text for details). Fractions were assayed for uronate by the modified carbazole method (●). The 1% (w/v)-agarose-gel electrophoretogram (inset) displays the electrophoretic migration of the material from pools A→C and the unfractionated A1D1 preparation. The direction of electrophoretic migration is indicated. (*b*) Electron micrographs of proteoglycans from (i) fraction A and (ii) fraction C spread on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon at an angle of 15°. (*c*) Length distribution histograms expressed as a frequency $[f(N_i) = N_i/\Sigma_i N_i]$ of molecules in fraction A (stippled bars) and fraction C (open bars). For those cases where the molecules occurred as blobs (without tails) the diameter was taken as a measure of their length.

distribution of particle sizes for fraction C can be seen in Fig. 4(*b*,ii). The number distributions for fractions A and C are shown in Fig. 4(*c*), and it may be concluded that column chromatography does not yield a useful separation of large from small proteoglycans on an analytical scale and thus is of limited use for preparative fractionation.

Isopycnic density-gradient separation

Several workers (Bonnet *et al.*, 1980; Buckwalter &

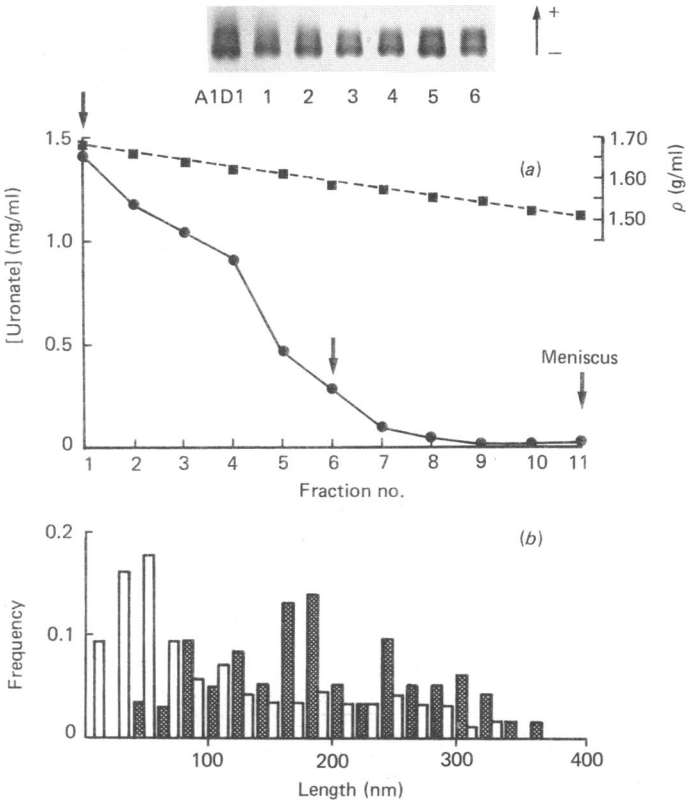

Fig. 5. Proteoglycan fractionation by isopycnic density-gradient centrifugation

(*a*) 4 M-Guanidinium chloride/CsCl density-gradient centrifugation profile of [^{14}C]proteoglycan (A1D1) (see the text for details). Fractions were analysed for uronate by the modified carbazole method (●) and density (■). The 1% (w/v)-agarose-gel electrophoretogram (inset) displays the electrophoretic migration of the material in fractions 1–6 and the total proteoglycan population (A1D1). The direction of electrophoretic migration is indicated. (*b*) Length distribution histograms, expressed as a frequency [$f(N_i) = N_i/\Sigma_i N_i$], of molecules in fraction 1 (stippled bars) and fraction 6 (open bars). In those cases where the molecules occurred as blobs (without tails) the diameter was taken as a measure of their length.

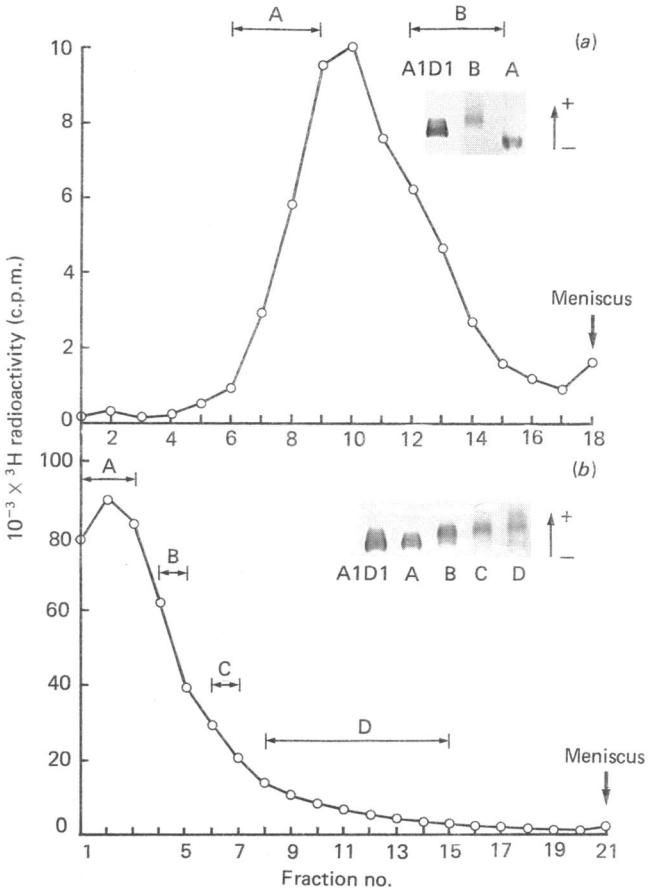

Fig. 6. Rate-zonal centrifugation of [^{3}H]proteoglycans (A1D1)

(*a*) Analytical rate-zonal centrifugation profile of [^{3}H]proteoglycans (○) in 4 M-guanidinium chloride/CsCl (ρ = 1.1–1.4 g/ml); for experimental details, see the text. The 1% (w/v)-agarose-gel electrophoretogram (inset) is of material from fractions A, B and the total proteoglycan population (A1D1). The direction of electrophoretic migration is indicated. (*b*) Preparative rate-zonal centrifugation profile of [^{3}H]proteoglycans (○) in 4 M-guanidinium chloride/CsCl (ρ = 1.15–1.45 g/ml); for experimental details, see the text. The 1% (w/v)-agarose-gel electrophoretogram (inset) is of material from pools A–D and the total proteoglycan population (A1D1). The direction of electrophoretic migration is indicated.

Rosenberg, 1982; Roughley & White, 1983) have noted a fractionation effect within the preparative-isopycnic-density-gradient step used in the proteoglycan purification. Consequently, a second dissociative density-gradient step was performed (Fig. 5*a*). Fractions 1–6 were analysed by agarose-gel electrophoresis (Fig. 5*a*) and by electron microscopy. A marked increase in the number of small proteoglycans with decreasing density may be discerned (Fig. 5*b*), but a complete separation of either species was not achieved.

Rate-zonal centrifugation

Rate-zonal centrifugation on sucrose gradients has been used by Heinegård *et al.* (1985*a*) to fractionate bovine nasal-cartilage proteoglycans. Though effective, it has the disadvantage of adding a reagent (sucrose) that interferes with routine analysis methods for these molecules and is difficult to remove completely. For this reason rate-zonal centrifugation in mixed-salt gradients formed from 4 M-guanidinium chloride/CsCl was investigated. These gradients combine the advantages of a separation attributable to differences in both sedimentation rate and buoyant density. The distribution of proteoglycans in an analytical gradient after 3.5 h is shown in Fig. 6(*a*), and the agarose-gel electrophoresis analysis indicates that separation of large and small proteoglycans has been achieved. After 16 h in the preparative gradient the large molecules are concentrated on the bottom of the tube (Fig. 6*b*). Agarose-gel electrophoresis (Fig. 6*b*) and electron microscopy indicate that the length of the proteoglycan molecules decreases towards the meniscus (Fig. 7), those molecules

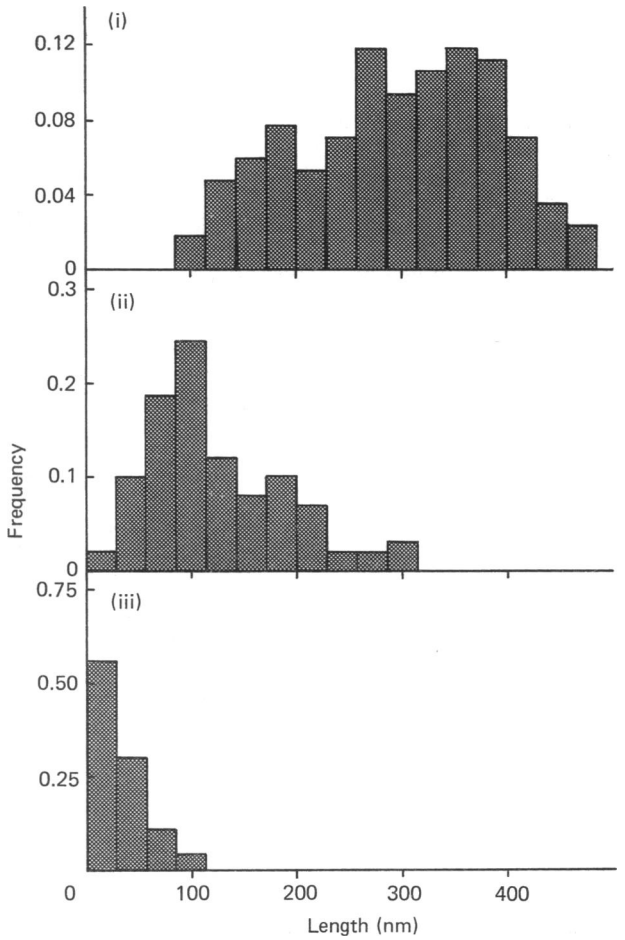

Fig. 7. Histograms of the length distributions of proteoglycans fractionated by rate-zonal centrifugation

Length distribution histograms expressed as a frequency $[f(N_i) = N_i/\Sigma_i N_i]$ of molecules in (i) fraction A, (ii) fraction C and (iii) fraction D. For those cases where the molecules occurred as blobs (without tails), the diameter was taken as a measure of their length.

in fraction D appearing predominantly as blobs and short rod-like structures.

It should be noted that there were no observable differences in the length distributions and proportions of 'blobs' for radio-labelled and non-radioactive proteoglycans.

DISCUSSION

These studies have employed BAC as a spreading agent rather than cytochrome *c*, which has been typically used by other workers. BAC was introduced in the DNA field by Koller *et al.* (1969) to obtain enhanced resolution of DNA and DNA–protein complexes and it appears to improve the resolution of proteoglycan images obtained by the spreading method. The larger proteoglycans have a beaded appearance, with approximately two to seven beads per proteoglycan. It is not clear whether this structure is an artefact or whether it represents some clustering of the chondroitin sulphate chains collapsed around the core. Many of the proteoglycans have a fine filamentous tail that we and other workers (Buckwalter & Rosenberg, 1982) believe to be the binding region end of the molecule. However, the technique does not resolve the two globular domains associated with the binding region when imaged by a rotary-shadowing replica technique as reported by Wiedemann *et al.* (1984). The micrographs of the total A1D1 population also show round compact structures that we have termed 'blobs', many of which also have a filamentous tail. These blobs were at first thought to be artefacts due to residual uranyl acetate stain not being removed by the washing procedure. However, they were not observed in fractionated preparations containing the larger proteoglycans, but they were greatly enriched in preparations of the smaller molecules. Thus the faster-migrating band observed in agarose-gel electrophoresis consisted almost entirely of particles staining in this way. The buoyant density ($\geqslant 1.58$ g/ml), gel-chromatographic and electrophoretic data all offer strong circumstantial evidence that these blobs are indeed proteoglycans or fragments thereof. The question arises as to whether this population appears as an artefact due to BAC or whether, as we believe, it was present in former work using cytochrome *c* (e.g. Buckwalter & Rosenberg, 1982).

Fractionation of the proteoglycans by size using gel chromatography is not very effective as monitored by agarose-gel electrophoresis and electron microscopy. The reason for this poor fractionation of large and small proteoglycans is in part due to the branched nature of the molecules, though it may be that proteoglycans also tend to self-associate and/or bind to the gel matrix or undergo large fluctuations in their hydrodynamic volume.

In agarose-gel electrophoresis the basis of the separation appears to be chiefly size, and this is consistent with the work of Heinegård *et al.* (1985*a*) and with the results in the DNA field. Whereas the fast-migrating proteoglycans appear to be uniformly small, the slower-migrating band, though dominated by large molecules, does contain the whole range of sizes, and it seems likely that the same factors which pertain to gel-filtration apply.

Isopycnic-gradient centrifugation has a marked fractionation effect such that smaller proteoglycans tend to have lower buoyant densities. This has been noted for bovine nasal-cartilage proteoglycans (Buckwalter & Rosenberg, 1982). Conversely, the larger proteoglycans are enriched at higher density. However, this principle alone does not give a satisfactory separation of large and small molecules.

Since the proteoglycans with higher buoyant density also have the higher sedimentation rate (Heinegård *et al.*, 1985*a*), and vice versa, gradients combining both principles were designed. Rate-zonal centrifugation in a 4 M-guanidinium chloride/CsCl gradient (ρ 1.15–1.45 g/ml) yielded the most effective fractionation as monitored by agarose-gel electrophoresis and electron microscopy.

We thank Mrs. R. Berry for the photography, and the Arthritis and Rheumatism Council (I.A.N.), the Medical Research Council (I.A.N.) and the Cystic Fibrosis Research Trust (J.K.S.) for support.

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Received 17 April 1986/24 June 1986; accepted 22 July 1986