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The properties of proteoglycan prepared from human articular cartilage by using associative caesium chloride gradients of high and low starting densities

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Proteoglycan was extracted from adult human articular cartilage from both the knee and the hip, and A1 preparations were prepared by CsCl-density-gradient centrifugation at starting densities of 1.69 and 1.5 g/ml. Irrespective of whether the cartilage was diced to 1 mm cubes or sectioned to 20 μm slices there was always a lower proportion of both protein and proteoglycan aggregate in the A1 preparation prepared at 1.69 g/ml. Furthermore, the addition of exogenous hyaluronic acid to the extracts before centrifugation did not improve the yield of aggregate at 1.69 g/ml. These results were not affected by the presence of proteinase inhibitors in the extraction medium. It appears that adult human articular cartilage contains a high proportion of low-density proteoglycan subunits and hyaluronic acid-binding proteins that make most of the re-formed proteoglycan aggregates of a lower density than is usually encountered with younger human and mammalian hyaline cartilages.

INTRODUCTION

Large proteoglycan molecules containing numerous chondroitin sulphate and keratan sulphate chains are a major component of most hyaline cartilage. In articular cartilage these molecules are thought to confer the tissue with an ability to withstand the compressive forces encountered during weight-bearing (Kempson *et al.*, 1976). The proteoglycan molecules do not exist in isolation, but occur as multi-molecular aggregates in which many proteoglycan subunits interact with a hyaluronic acid molecule (Hardingham & Muir, 1972; Hascall, 1977) through one end of their core protein (Heinegård & Hascall, 1974; Hardingham *et al.*, 1976), and each interaction may be stabilized by the further interaction of a link protein (Hardingham, 1979; Franzen *et al.*, 1981). In the tissue such proteoglycan aggregates may also interact with the fibrillar collagenous network (Poole *et al.*, 1982) that comprises the bulk of the extracellular matrix.

The isolation of proteoglycans and other non-collagenous molecules from the cartilage matrix is usually performed by the use of dissociating solvents such as 4 M-guanidinium cloride or 2 M-$CaCl_2$ (Sajdera & Hascall, 1969; Mason & Mayes, 1973), and proteinase inhibitors are often included to prevent proteolysis (Oegema *et al.*, 1975; Dickson & Roughley, 1978). The proteoglycans can then be separated from other proteins by CsCl-density-gradient centrifugation by virtue of their high buoyant density in this medium. If conditions that permit the formation of the proteoglycan aggregate are used, the intact aggregate can be recovered at the bottom of the gradient, whereas when dissociative conditions are used the proteoglycan subunits, hyaluronic acid and link proteins can be separated because of their respectively decreasing buoyant densities.

Traditionally, a starting density of about 1.7 g/ml was used in the preparation of proteoglycan aggregates. This works well in bovine nasal and young human articular cartilages (Roughley *et al.*, 1984), with the proteoglycan aggregate sedimenting to the bottom of the gradients. However, in the adult human this is not the case, and a poor yield of aggregate is recovered from the bottom of the gradient (Roughley *et al.*, 1984). Proteoglycan aggregates can be prepared from this tissue if the starting density for centrifugation is lowered to 1.5 g/ml (Bayliss & Ali, 1978; Bayliss *et al.*, 1983). The reason for this discrepancy is presumably the lower density of the proteolygcan subunits in the adult human, caused by partial proteolytic cleavage of the molecules during their maturation in the extracellular matrix. There is evidence that in many cases this proteolytic modification proceeds to such an extent that only the hyaluronic acid-binding region of the proteoglycan subunit remains bound to the hyaluronic acid (Roughley *et al.*, 1984).

In the present paper we report the behaviour of the proteoglycan, extracted from various adult human articular cartilages by a variety of techniques, on CsCl-density-gradient centrifugation with starting densities of 1.69 and 1.5 g/ml. In addition, the effect of proteinase inhibitors in the extraction medium was also studied to determine whether endogenous enzymes released during the preparation could contribute to the proteolytic modification of the proteoglycans.

METHODS

Materials

Guanidinium chloride, hyaluronic acid, agarose, iodoacetamide and phenylmethanesulphonyl fluoride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Abbreviation used: SDS, sodium dodecyl sulphate.

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CsCl, Toluidine Blue and 3,3′,4,4′-tetra-aminobiphenyl hydrochloride were from BDH Chemicals (Poole, Dorset, U.K.). SDS, acrylamide, methylenebisacrylamide, Coomassie Brilliant Blue R250 and nitrocellulose sheets were from Bio-Rad Laboratories (Mississauga, Ont., Canada). Pepstatin was from the Peptide Institute Protein Research Foundation (Osaka, Japan). Sepharose CL-2B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Sheep anti-(human link protein) IgG and pig anti-[sheep $F(ab')_2$] IgG conjugated with peroxidase were prepared as described previously (Roughley *et al.*, 1985).

Source of cartilage

Adult human articular cartilage was obtained from the knee (femoral condyles) or the hip (femoral head) at the time of autopsy or surgery. All tissue used appeared macroscopically normal. The tissue was prepared for extraction either by dicing into pieces of about 1 mm^3 or by cutting 20 μm sections.

Extraction of proteoglycan

Proteoglycan was extracted from the cartilage with 4 M-guanidinium chloride/0.1 mM-sodium acetate, pH 6.0, at 10 ml/g wet wt. of cartilage. Extraction was for 68 h at 4 °C with continuous stirring. In some cases the extraction fluid was supplemented with proteinase inhibitors: EDTA, phenylmethanesulphonyl fluoride and iodacetamide each at 1 mM and pepstatin at 10 μg/ml. The extracts were filtered through glass wool, and the filtrates were dialysed against 0.1 M-sodium acetate, pH 6.0, at 4 °C for 24 h to allow reassociation of the proteoglycan aggregate. For the preparation from diced cartilage, hyaluronic acid was sometimes added to the filtered extract at 50 μg/ml before dialysis.

Density-gradient centrifugation

The dialysed cartilage extracts were adjusted to 1.69 or 1.50 g/ml by the addition of solid CsCl (1.28 and 0.84 g/ml of extract respectively). The solutions were then centrifuged at 100000 $g_{av.}$ at 10 °C for 48 h. Upon termination, the resulting gradients were fractionated, and the fractions assayed for density, uronic acid content (Bitter & Muir, 1962) and A_{280}. In addition, the fractions were analysed for proteoglycan content by agarose/polyacrylamide-gel electrophoresis, and for protein content by SDS/polyacrylamide-gel electrophoresis.

Sepharose CL-2B chromatography

Proteoglycan recovered as an A1 preparation from the bottom of the gradients (density greater than 1.73 or 1.54 g/ml, depending on starting conditions) was chromatographed through Sepharose CL-2B (1 cm × 110 cm) at a flow rate of 6 ml/h. Hyaluronic acid [2% (w/w), based on uronic acid content] was mixed with some A1 fractions before chromatograpy. The elution buffer was 0.2 M-sodium acetate, pH 5.5, and the resulting fractions were assayed for uronic acid and protein content. In some cases the proteoglycan sedimenting at lower density (A2 preparation; density 1.73–1.66 g/ml or 1.54–1.48 g/ml, depending on starting conditions) was chromatographed in a similar manner.

Agarose/polyacrylamide-gel electrophoresis

Composite gels of 0.6% agarose and 1.2% acrylamide were prepared by the method of McDevitt & Muir (1971), with 10 mM-Tris/HCl, pH 7.5, as both the gel buffer and electrophoresis buffer. Samples comprised fractions from the density gradients dialysed against water and then mixed with an equal volume of 50% (w/v) sucrose

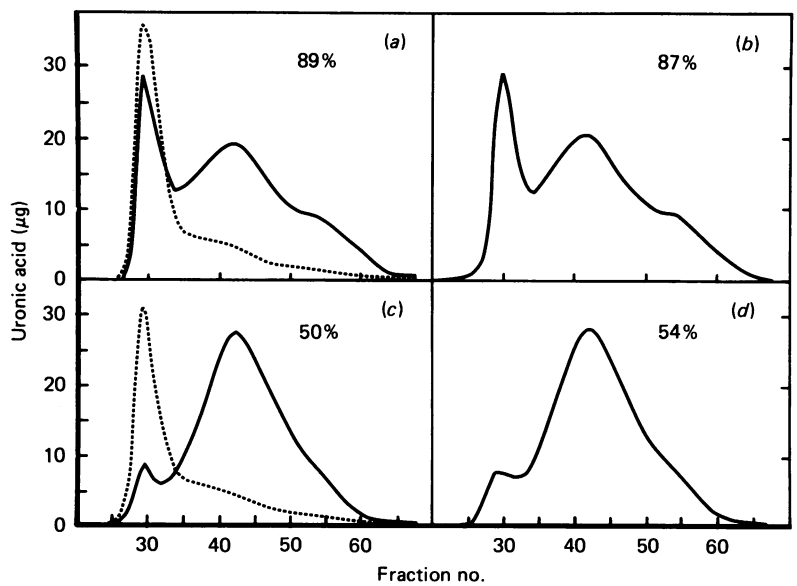

Fig. 1. Sepharose CL-2B chromatography of A1 preparations from extracts of diced hip cartilage

Adult human hip cartilage was diced, then extracted with 4 M-guanidinium chloride in the presence (*a* and *c*) or absence (*b* and *d*) of proteinase inhibitors. Extracts were dialysed, then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.5 g/ml (*a* and *b*) or 1.69 g/ml (*c* and *d*). The resulting A1 preparations were analysed by chromatography through Sepharose CL-2B, in either the absence (——) or the presence (-----) of 2% hyaluronic acid. Fractions were monitored for uronic acid content. The percentage of uronic acid recovered in each A1 fraction is also shown.

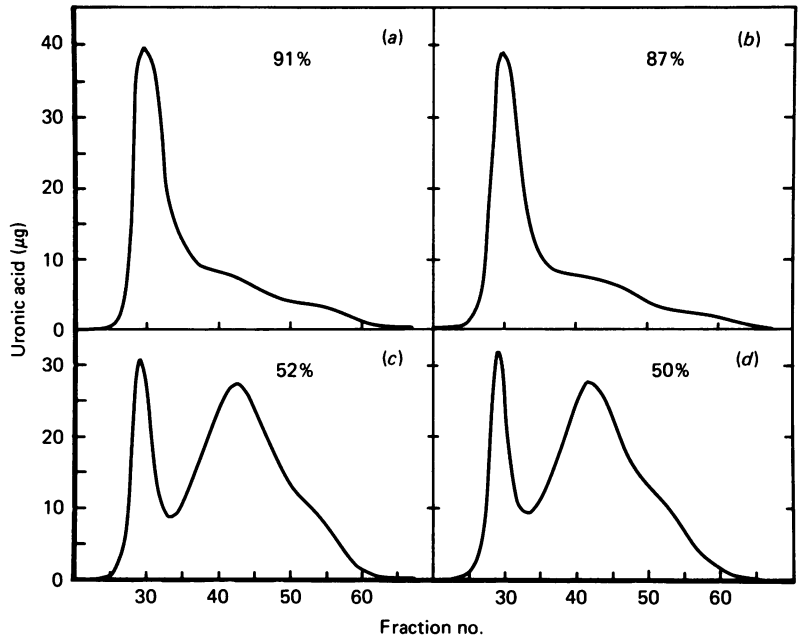

Fig. 2. Sepharose CL-2B chromatography of A1 preparations from extracts of diced hip cartilage containing added hyaluronic acid

Adult human hip cartilage was diced, then extracted with 4 M-guanidinium chloride in the presence (*a* and *c*) or absence (*b* and *d*) of proteinase inhibitors. Exogenous hyaluronic acid was added to the extracts, which were dialysed, then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.5 g/ml (*a* and *b*) or 1.69 g/ml (*c* and *d*). The resulting A1 preparations were analysed by chromatography through Sepharose CL-2B. Fractions were monitored for uronic acid content.

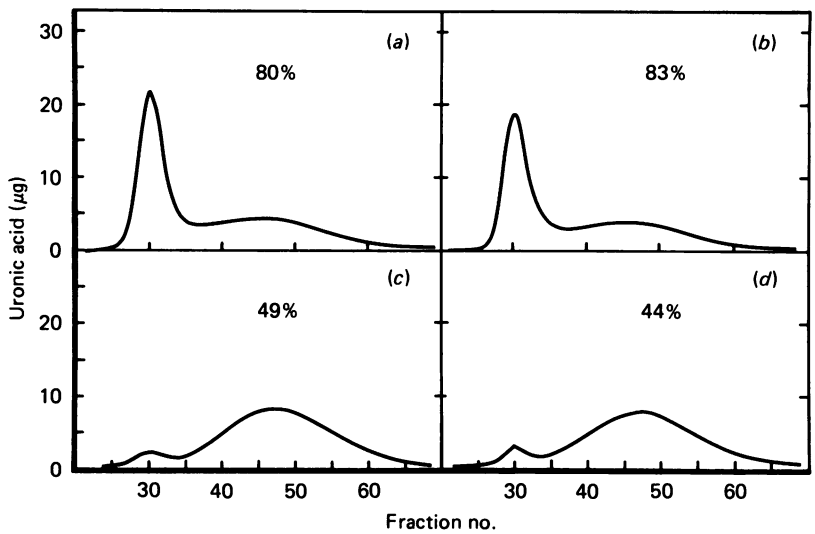

Fig. 3. Sepharose CL-2B chromatography of A1 preparations from extracts of diced knee cartilage containing added hyaluronic acid

Adult human knee cartilage was diced, then extracted with 4 M-guanidinium chloride in the presence (*a* and *c*) or absence (*b* and *d*) of proteinase inhibitors. Exogenous hyaluronic acid was added to the extracts, which were dialysed, then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.5 g/ml (*a* and *b*) or 1.69 g/ml (*c* and *d*). The resulting A1 preparations were analysed by chromatography through Sepharose CL-2B. Fractions were monitored for uronic acid content.

containing 0.005% Bromophenol Blue. Electrophoresis was initially at 1 mA/gel until the sample had entered and then at 4 mA/gel until the dye had migrated 3 cm into the gel. The gel was stained with 0.1 M-acetic acid containing 0.02% Toluidine Blue, and destained at 35 °C with 0.5 M-acetic acid.

SDS/polyacrylamide-gel electrophoresis

Fractions from the density gradients were dialysed against 0.125 M-Tris/HCl, pH 6.8, containing 0.1% SDS. Before electrophoresis, samples were mixed with an equal volume of the same buffer containing 2% SDS, 1%

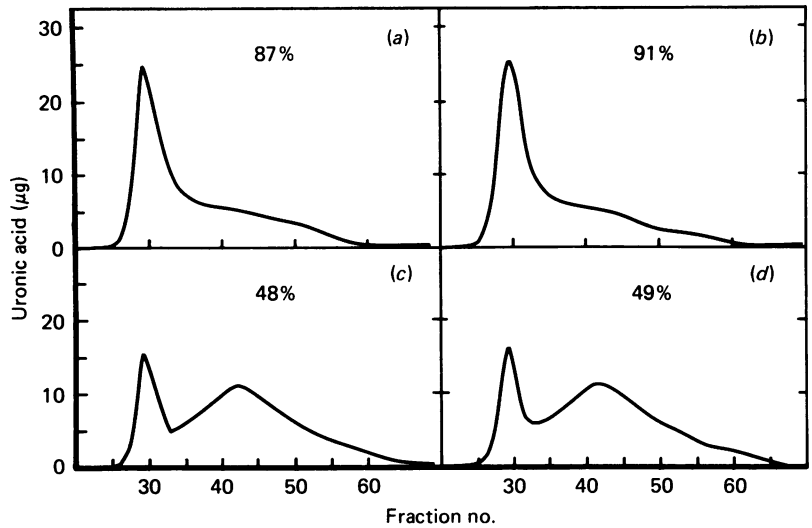

Fig. 4. Sepharose CL-2B chromatography of A1 preparations from extracts of sectioned hip cartilage

Adult human hip cartilage was sectioned to 20 μm, then extracted with 4 M-guanidium chloride in the presence (*a* and *c*) or absence (*b* and *d*) of proteinase inhibitors. Extracts were dialysed, then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.5 g/ml (*a* and *b*) or 1.69 g/ml (*c* and *d*). The resulting A1 preparations were analysed by chromatography through Sepharose CL-2B. Fractions were monitored for uronic acid content.

glycerol, 0.001% Bromophenol Blue and 5% (v/v) mercaptoethanol, then heated at 100 °C for 3 min. Electrophoresis was performed in 10%-polyacrylamide slab gels by the discontinuous method of Laemmli (1970). After electrophoresis, proteins were either stained with Coomassie Brilliant Blue R250 (Fairbanks *et al.*, 1971) or transferred to nitrocellulose for immunodetection (Towbin *et al.*, 1979). Link proteins were identified by indirect immune staining as described previously (Roughley *et al.*, 1982) by using first a sheep anti-(human link protein) IgG and then a pig anti-[sheep $F(ab')_2$] IgG conjugated with peroxidase.

RESULTS

When proteoglycans were extracted from diced hip cartilage, the A1 preparations obtained by using either 1.69 or 1.5 g/ml as the starting density showed only a low proportion of aggregate (Fig. 1), though more aggregate was obtained at the lower starting density. Chromatography of the A1 preparations in the presence of hyaluronic acid indicated that most of the proteoglycan could bind to hyaluronic acid, and that the lack of aggregate in the preparations was probably due to the inability to extract hyaluronic acid from the cartilage (Bayliss *et al.*, 1983). It was also apparent that the presence or absence of proteinase inhibitors in the extraction medium did not affect the chromatography profile, indicating that the broad profiles reflected proteoglycan distribution within the tissue rather than heterogeneity created by proteolysis during extraction and preparation.

To compensate for the absence of hyaluronic acid in the extracts of diced cartilage, exogenous hyaluronic acid was added to the extracts before centrifugation. In this case the A1 preparation prepared by using a low starting density showed a high proportion of aggregate (Fig. 2). However, the use of the high starting density still resulted in little aggregate formation. A similar result was obtained when diced knee cartilage was used for extraction (Fig. 3), though in this case the absence of aggregate when the high starting density was used was even more pronounced, consistent with earlier work (Roughley *et al.*, 1984). Once again the presence of proteinase inhibitors did not affect the results.

An additional way of ensuring that hyaluronic acid is present in the extracts is to use 20 μm sections of cartilage (Bayliss *et al.*, 1983). Under these conditions most of the endogenous hyaluronic acid is extracted together with most of the proteoglycan. However, when A1 preparations were prepared from these extracts, the result was no different from that obtained with the diced cartilage extract in the presence of exogenous hyaluronic acid (Fig. 4), even though a greater concentration of hyaluronic acid might be expected to be present. Furthermore, even under the conditions of sectioning, where cell lysis and proteinase release may occur, there was no beneficial effect on the profiles by the inclusion of proteinase inhibitors in the extraction fluid.

The difference in proteoglycan sedimentation attributable to the different starting densities was further investigated by analysing the distribution of macromolecules throughout the gradients obtained from extracts in the presence of added hyaluronic acid. When the low starting density was used, most of the proteoglycan sedimented as a viscous solution to the bottom of the gradient, with a density greater than 1.55 g/ml (Fig. 5*b*). With the high starting density much of the proteoglycan sedimented with a density greater than 1.75 g/ml, though there was also a reasonable amount of material sedimenting in a more viscous solution at the top of the gradient with a density less than 1.65 g/ml (Fig. 5*a*). The distribution of protein was also different for the two starting densities. With the high starting density, most of the protein was recovered at the top of the gradient (Fig. 5*a*), whereas with the low starting density there was more protein associated with the proteoglycan at high density

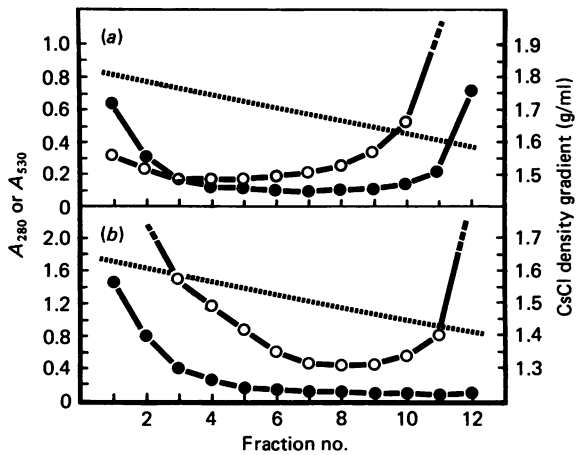

Fig. 5. CsCl-density-gradient centrifugation of extracts from diced knee cartilage

Adult human knee cartilage was diced, then extracted with 4 M-guanidinium chloride in the presence of proteinase inhibitors. Exogenous hyaluronic acid was added to the extract, which was dialysed and then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.69 g/ml (*a*) or 1.5 g/ml (*b*). Fractions were monitored for density (----), uronic acid content (●; A_{530}, carbazole assay) and A_{280} (○).

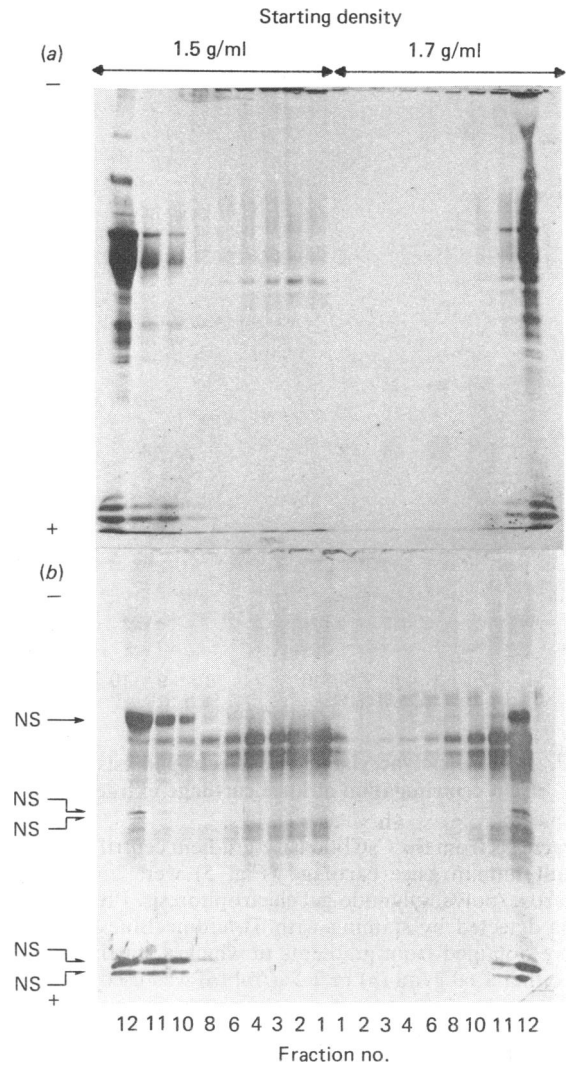

Fig. 6. SDS/polyacrylamide-gel electrophoresis of fractions from centrifugation of knee cartilage extracts

Fractions from CsCl-density-gradient centrifugation of adult human knee cartilage (Fig. 5) were analysed by SDS/polyacrylamide-gel electrophoresis. Protein was detected either directly by staining with Coomassie Brilliant Blue (*a*) or by immune localization by using an antiserum to link proteins after transfer to nitrocellulose (*b*). NS indicates proteins which stain non-specifically with the immune-localization procedure. The order of the fractions for the two gradients on the gel is from the centre outwards.

than at the top of the gradient (Fig. 5*b*). This suggests that much of the protein in the cartilage extract can interact with hyaluronic acid.

The distribution of cartilage proteins was also examined by SDS/polyacrylamide-gel electrophoresis (Fig. 6). With both starting densities most of the proteins were recovered at low density at the top of the gradients. The only exception appears to be the link proteins and a diffuse material of about 70000 M_r that were recovered with the proteoglycan at the bottom of the gradient when a low starting density was used. The distribution of proteoglycan in the gradients was examined by agarose/polyacrylamide-gel electrophoresis (Fig. 7). When the low starting density was used, all proteoglycan appeared at the bottom of the gradient, together with the hyaluronic acid, which migrates more slowly on electrophoresis. With the high starting density, proteoglycan was apparent at both extremes of the gradient, as expected, though hyaluronic acid was only apparent in the low-density fractions. Irrespective of the density at which it sediments, the proteoglycan which is stained with Toluidine Blue appears to be of the same band mobility.

If the hyaluronic acid sediments at low density when the high starting density is used, then one might expect that the proteoglycan isolated as an A2 preparation (density less than 1.73 g/ml) from this gradient would appear in a mainly aggregated form on Sepharose CL-2B chromatography, and this was indeed found to be the case (Fig. 8). It is also apparent that the aggregate in this preparation is associated with a considerable amount of protein, in common with the preparations obtained with the low starting density, but in contrast with the A1 preparation obtained with the high starting density, which is mainly in a subunit form. Thus, in preparations from adult human cartilage, proteoglycan aggregates are always associated with large amounts of protein which binds to hyaluronic acid.

DISCUSSION

We have shown that, if a starting density of about 1.69 g/ml is used in the preparation of 'proteoglycan aggregate' from adult human articular cartilage by CsCl-density-gradient centrifugation under associative conditions, then mainly proteoglycan subunits are recovered at the bottom of the gradient. This is irrespective of whether the cartilage was diced to 1 mm cubes or sectioned to 20 μm slices, or whether the extracts

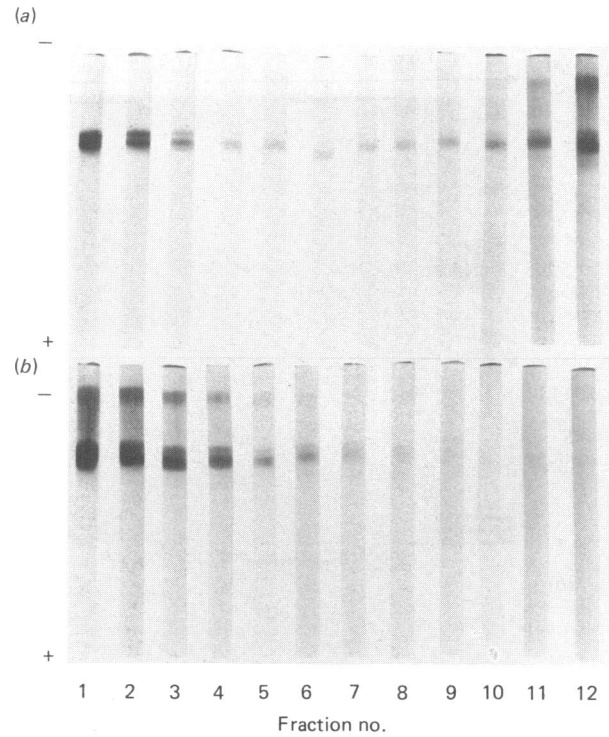

Fig. 7. Agarose/polyacrylamide-gel electrophoresis of fractions from centrifugation of knee cartilage extracts

Fractions from the CsCl-density-gradient centrifugation of adult human knee cartilage (Fig. 5) were analysed by agarose/polyacrylamide-gel electrophoresis. Proteoglycan was detected by staining with Toluidine Blue. Fractions were obtained from gradients in which a starting density of either 1.69 g/ml (*a*) or 1.5 g/ml (*b*) was used.

were supplemented with hyaluronic acid. In contrast, if a starting density of 1.5 g/ml is used the proteoglycan is all recovered as aggregate at the bottom of the gradient. This aggregate contains link proteins, as expected. A similar aggregate also appears to be present at the top of the high-density gradient.

The reason for this phenomenon would appear to be the high proportion of protein present in the proteoglycan aggregate, which lowers the density much below that encountered for aggregates from young animal or human cartilage. This high protein content is due not entirely to the increased protein content of the subunits themselves, but rather to the presence of a considerable amount of other protein in association with the aggregate. This is consistent with previous observations (Roughley *et al.*, 1984, 1985) that adult human articular cartilage contains a large amount of a hyaluronic acid-binding protein, probably derived from the hyaluronic acid-binding region of the proteoglycan subunits. The presence of this material necessitates the use of a low starting density if one wishes to prepare proteoglycan aggregates from adult human articular cartilage.

The presence of this hyaluronic acid-binding protein and the small size and high protein content of the proteoglycan subunit in the adult cartilage suggest that proteolytic modification of the proteoglycan is taking place within the extracellular matrix of the tissue. The observed fragmentation of the link proteins with increasing age (Mort *et al.*, 1983) would be compatible with this view. It could, however, be argued that proteolysis was occurring during the extraction procedure, though the inability of proteinase inhibitors to affect the size of the proteoglycan subunits or their ability to interact with hyaluronic acid would tend to preclude this view. Furthermore, link-protein fragmentation is also unaffected by the absence or presence of proteinase inhibitors (results not shown). It therefore appears that, as long as care is taken to keep the temperature around

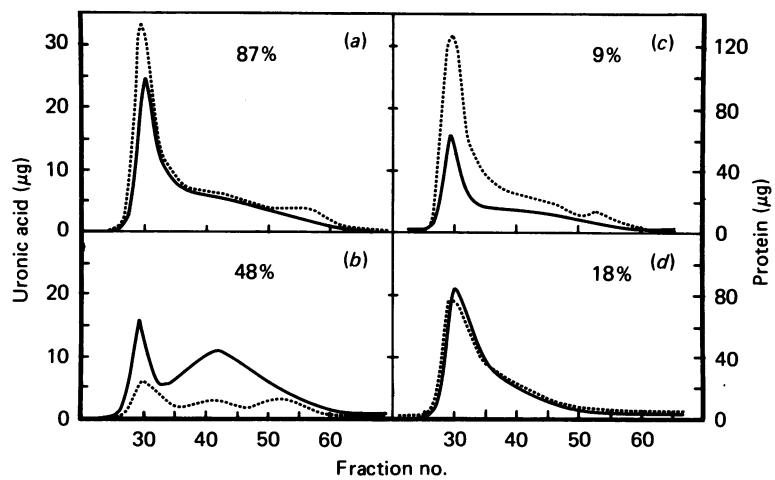

Fig. 8. Sepharose CL-2B chromatography of A1 and A2 preparations from extracts of sectioned hip cartilage

Adult human hip cartilage was sectioned at 20 μm, then extracted with 4 M-guanidinium chloride in the presence of proteinase inhibitors. The extract was dialysed and then subjected to CsCl-density-gradient centrifugation under associative conditions by using a starting density of either 1.5 g/ml (*a* and *b*) or 1.69 g/ml (*c* and *d*). The resulting A1 (*a* and *c*) and A2 (*b* and *d*) preparations were analysed by chromatography through Sepharose CL-2B. Fractions were monitored for uronic acid (——) and protein content (----).

4 °C during preparation, it is not necessary to use proteinase inhibitors to prevent degradation during the preparation of proteoglycan from this tissue. In fact, one might argue that inhibitors such as iodoacetamide, which can potentially modify all thiol groups, may cause structural changes that could interfere with the analysis of cartilage macromolecules. Perhaps, therefore, inhibitors of this type should only be used if it is known that their inclusion is essential.

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