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Identification of a hyaluronic acid-binding protein that interferes with the preparation of high-buoyant-density proteoglycan aggregates from adult human articular cartilage

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Adult human articular cartilage contains a hyaluronic acid-binding protein of M_r 60000–75000, which contains disulphide bonds essential for this interaction. The molecule can compete with proteoglycan subunits for binding sites on hyaluronic acid, and can also displace proteoglycan subunits from hyaluronic acid if their interaction is not stabilized by the presence of link proteins. The abundance of this protein in the adult accounts for the reported inability to prepare high-buoyant-density proteoglycan aggregates from extracts of adult human cartilage [Roughley, White, Poole & Mort (1984) Biochem. J. **221**, 637–644], whereas the deficiency of the protein in newborn human cartilage allows the normal recovery of proteoglycan aggregates from this tissue. The protein shares many common features with a hyaluronic acid-binding region derived by proteolytic treatment of a proteoglycan aggregate preparation, and this may also represent its origin in the cartilage, with its production increasing during tissue maturation.

INTRODUCTION

The proteoglycans of hyaline cartilage have the ability to interact specifically with hyaluronic acid to form large aggregates (Hardingham & Muir, 1972). These proteoglycan aggregates consist of many proteoglycan subunits linked to a central filament of hyaluronic acid by one terminus of their core protein (Hascall, 1977). This hyaluronic acid-binding region is devoid of the chondroitin sulphate and keratan sulphate chains that characterize the remainder of the proteoglycans (Heinegård & Hascall, 1974), and may represent the major site for the attachment of *N*-linked oligosaccharides (Lohmander *et al.*, 1980). In contrast, *O*-linked oligosaccharides appear to be more abundant throughout the remainder of the core protein of the proteoglycan subunits (Lohmander *et al.*, 1980). The interaction between the proteoglycan subunits and hyaluronic acid is stabilized by the further interaction of a link protein (Hardingham, 1979; Tang *et al.*, 1979; Franzen *et al.*, 1981). A single link protein molecule may bind to the hyaluronic acid-binding region of each proteoglycan subunit and the adjacent hyaluronic acid in the aggregate (Oegema *et al.*, 1977; Faltz *et al.*, 1979; Poole *et al.*, 1980*b*).

It has been shown that this molecular organization also occurs *in situ* within the extracellular matrix for both bovine and human articular cartilages (Poole *et al.*, 1982). This type of organization was not detectable throughout the whole matrix, but was confined to the interterritorial matrix of middle and deep zones. In these regions the hyaluronic acid filaments appeared to stretch between the collagen fibrils to form a three-dimensional network. Moreover, the hyaluronic acid molecules appeared to be anchored to the collagen fibrils. It is this type of proteoglycan network that has been postulated to be a major factor in permitting articular cartilage to resist reversibly the compressive forces encountered under load (Hascall, 1977; Roughley, 1982). One may therefore envisage that any parameter that interferes with the formation of stable proteoglycan aggregates will be detrimental to the functional properties of the articular cartilage.

In a previous paper we showed that, although it is possible to prepare high-buoyant-density proteoglycan aggregates from neonatal human articular cartilage, one cannot prepare their counterparts from adult human human articular cartilage (Roughley *et al.*, 1984). A similar low recovery of proteoglycan aggregate has also been reported for adult human costal cartilage (Pearson & Mason, 1979). In such cases the extracts have been shown to contain link proteins, hyaluronic acid and proteoglycan subunits with functional hyaluronic acid-binding regions. It became apparent that the inability to prepare the adult proteoglycan aggregates was due to the presence of a low-buoyant-density component, unique in its abundance to the adult cartilage, which presumably competed with the proteoglycan subunits for binding sites on the hyaluronic acid molecules. In the present study we describe the characterization of this component and discuss its relationship to an isolated hyaluronic acid-binding region derived from a proteoglycan subunit by proteolytic cleavage.

METHODS

Materials

Guanidinium chloride and hyaluronic acid (from human umbilical cord) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and CsCl and 3,4,3′,4′-tetraaminobiphenyl tetrahydrochloride were from BDH Chemicals (Montreal, Que., Canada). The hyaluronic acid was further purified by precipitation with cetylpyridinium chloride by using the procedure described by Cleland & Sherblom (1977). Sodium dodecyl sulphate, acrylamide, methylenebisacrylamide, Coomassie Brilliant Blue R250, Stains All and nitrocellulose sheets were from Bio-Rad Laboratories (Mississauga, Ont., Canada).

Sepharose CL-6B and CL-2B were from Pharmacia Fine Chemicals (Montreal, Que., Canada).

Extraction of cartilage

Human articular cartilage was obtained from the knees of neonatal infants and adults at the time of autopsy. In all cases autopsy was performed within 20 h of death, and only cartilage that appeared macroscopically normal was taken. Cartilage was finely diced with a scalpel to pieces with dimensions of about 1 mm^3, and then extracted with 10 vol. of 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0, at 4 °C with continuous stirring for 48 h. The extraction fluid also contained the proteinase inhibitors iodoacetamide (1 mM), EDTA (1 mM), phenylmethanesulphonyl fluoride (1 mM) and pepstatin (5 μg/ml) (Roughley & White, 1980). The extract was then separated from the cartilage residue by filtration through glass-wool.

Isolation of proteoglycan and protein preparations

The cartilage extracts were subjected to CsCl-density-gradient centrifugation under either associative or dissociative conditions (Hascall & Sajdera, 1969). For associative conditions the extracts were supplemented with 50 μg of hyaluronic acid/ml and then dialysed for 24 h at 4 °C against 100 vol. of 0.1 M-sodium acetate buffer, pH 6.0, and then adjusted to a density of 1.50 g/ml by the addition of CsCl (0.8 g/ml). For dissociative conditions CsCl (0.8 g/ml) and guanidinium chloride (0.23 g/ml) were added directly to the extract to give a final density of 1.50 g/ml. Centrifugation under both associative and dissociative conditions was performed at 100000 $g_{av.}$ for 48 h at 10 °C. Gradients were then fractionated for the measurement of density, uronic acid (Bitter & Muir, 1962) and absorbance at 280 nm. Proteoglycan aggregates were obtained as an A1 preparation (from associative conditions) with a density greater than 1.55 g/ml, and cartilage proteins that did not bind to hyaluronic acid were obtained as an A2 preparation with a density less than 1.55 g/ml. Proteoglycan subunits were obtained as a D1 preparation (from dissociative conditions) with a density greater than 1.54 g/ml, and other cartilage proteins were obtained as a D3 preparation with a density less than 1.44 g/ml. Alternatively, proteoglycan aggregate preparations were dissolved in 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0, containing CsCl to give a density of 1.50 g/ml, and subjected to centrifugation as above. In this case proteoglycan subunits were obtained as an A1D1 preparation with a density greater than 1.54 g/ml, and cartilage proteins having an affinity for hyaluronic acid were obtained as an A1D3 preparation with a density less than 1.44 g/ml. In all cases, preparations were freeze-dried after conversion into their potassium salts by dialysis against 0.1 M-potassium acetate buffer, pH 6.0, and subsequent exhaustive dialysis against water.

Sepharose CL-6B chromatography

Samples of D3 and A1D3 preparations were dissolved in 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5, at 8 mg/ml. Samples (5 ml) were than applied to a Sepharose CL-6B column (80 cm × 2.2 cm) and eluted at 16 ml/h. The column equilibration and elution buffers were also 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5. The resulting fractions (4–5 ml) were assayed for uronic acid, absorbance at 280 nm and protein content by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Fractions were pooled for use in recombination experiments as indicated in the Figures, and pools were either concentrated through an Amicon YM5 ultrafiltration membrane or were dialysed and freeze-dried as described for the initial preparations. The void and total volumes of the column were determined by the elution of Blue Dextran and $K_3Fe(CN)_6$ respectively.

Reduction and alkylation

D3 preparations were dissolved at 4 mg/ml in 4 M-guanidinium chloride / 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-dithiothreitol and incubated at 40 °C for 5 h. Iodoacetamide was then added to a concentration of 20 mM, and the solution was incubated for a further 1 h at 40 °C and then for 20 h at 4 °C (Heinegård, 1977). After dialysis to remove the reduction and alkylation reagents, the solutions were used directly in recombination experiments.

Recombination experiments

Various combinations of proteoglycan subunits, cartilage protein preparations and hyaluronic acid were dissolved in 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0. The mixtures were then dialysed to associative conditions as described previously and supplemented with CsCl at 1.2 g/ml to give a density of 1.68 g/ml. After centrifugation, A1 preparations having a density greater than 1.72 g/ml were isolated. Unless otherwise stated, recombination experiments were performed with proteoglycan, protein and hyaluronic acid concentrations of 1 mg/ml, 1 mg/ml and 20 μg/ml respectively.

Sepharose CL-2B chromatography

A1 preparations from recombination experiments were dialysed against 0.2 M-sodium acetate buffer, pH 5.5. Samples (1 ml) of the proteoglycan preparations were analysed by chromatography through a Sepharose CL-2B column (110 cm × 1 cm) with 0.2 M-sodium acetate buffer, pH 5.5, as the elution buffer at a flow rate of 6 ml/h. The resulting fractions (1 ml) were assayed for uronic acid content. The void and total volumes of the column were determined by the elution of proteoglycan aggregate (from bovine nasal cartilage) and glucurono-lactone respectively.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples were analysed by electrophoresis in 10% (w/v) polyacrylamide gels by the methods of Laemmli (1970). Freeze-dried preparations were dissolved at 2 mg/ml in 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% (w/v) sodium dodecyl sulphate, and samples from Sepharose CL-6B chromatography were dialysed against 400 vol. of the same buffer. Before electrophoresis, samples were mixed with an equal volume of 0.125 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate, 1% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue in the presence or in the absence of 5% (v/v) 2-mercaptoethanol and heated at 100 °C for 3 min. After electrophoresis proteins were either stained with Coomassie Brilliant Blue R250 by the method of Fairbanks *et al.* (1971) or with Stains All by the method of Green

et al. (1973). Alternatively, proteins were transferred to a nitrocellulose sheet for immunoidentification.

Electrophoretic transfer and immunoidentification

Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin *et al.* (1979). Link protein was then identified by indirect immune staining with the use of first a sheep anti-(neonatal human cartilage link protein) IgG and then a peroxidase-conjugated pig anti-[sheep $F(ab')_2$] IgG as described previously (Roughley *et al.*, 1982). Detection was by incubation with tetra-aminobiphenyl tetrahydrochloride and H_2O_2. Staining of the polyacrylamide gel with Coomassie Brilliant Blue after transfer showed that there was a total removal of protein from the areas corresponding to link protein.

Preparation of antisera and radioimmunossay

Sheep antibody to human link protein was raised against a native neonatal link protein preparation, purified from an A1D3 preparation by Sepharose CL-6B chromatography under dissociative conditions (Roughley *et al.*, 1982). The link protein was dissolved at 1 mg/ml in 0.5 M-NaCl, and injected intramuscularly as 1 ml, which contained the link protein emulsified with an equal volume of Freund's complete adjuvant, on day 0. On days 36 and 115 the injection was repeated with the antigen in Freund's incomplete adjuvant. The sheep was bled out on day 179. A pig antiserum to sheep IgG $F(ab')_2$ was prepared (Poole *et al.*, 1980*a*) and labelled with horseradish peroxidase (Champion & Poole, 1981) as described previously. The rabbit antiserum to a hyaluronic acid-binding region derived from Swarm rat chondrosarcoma proteoglycan by clostripain digestion was prepared as described by Kimura *et al.* (1981), and the mouse monoclonal antibody to keratan sulphate was prepared essentially as described by Caterson *et al.* (1983) and showed the same specificity towards keratan sulphate present on proteoglycans (C. Webber, P. J. Roughley & A. R. Poole, unpublished work). The procedure for radioimmunoassay with the use of ^{125}I-labelled proteoglycan subunit as the competing antigen and *Staphylococcus aureus* bearing protein A for precipitation of immune complexes was essentially as described by Christner *et al.* (1980).

RESULTS

In a previous paper (Roughley *et al.*, 1984) we demonstrated that the factor responsible for preventing the formation of high-buoyant-density proteoglycan aggregates from adult human articular cartilage resides with the low-density molecules isolated from the cartilage (D3 preparation). Initial work on the characterization of this component involved the reduction and alkylation of disulphide bonds present in the D3 preparation to determine whether a specific protein conformation was responsible for the effect. After treatment with dithiothreitol and iodoacetamide in the presence of 4 M-guanidinium chloride the D3 preparation was no longer able to prevent the formation of proteoglycan aggregates, when used in re-aggregation experiments with an adult D1 preparation and hyaluronic acid (Fig. 1).

In order to characterize this protein, the adult D3 preparation was fractionated on Sepharose CL-6B in the presence of 4 M-guanidinium chloride. The resulting

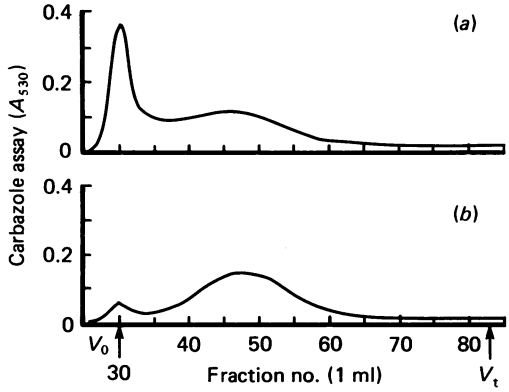

Fig. 1. Sepharose CL-2B chromatography of A1 preparations obtained by CsCl-density-gradient centrifugation of mixtures containing D1 preparations, D3 preparations and hyaluronic acid

A D1 preparation from adult human articular cartilage was mixed with hyaluronic acid and an adult D3 preparation in 4 M-guanidinium chloride. The D3 preparation was either (*a*) reduced and alkylated, or (*b*) untreated. Samples were dialysed to associative conditions before CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography. In this and subsequent Figures V_0 and V_t indicate void volume and total excluded volume respectively.

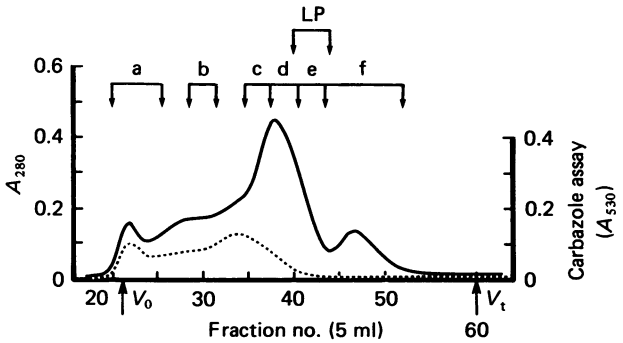

Fig. 2. Sepharose CL-6B chromatography of an adult D3 preparation under dissociative conditions

An adult D3 preparation was dissolved in 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5, and chromatographed in the same buffer through Sepharose CL-6B. Fractions were collected, monitored for absorbance at 280 nm (——) and uronic acid (· · · · ·) and then pooled for further investigation (a, b, c, d, e and f). The elution position of link proteins (LP) is also shown.

profile showed four regions of protein elution (Fig. 2). The void-volume peak and the first included peak contained uronic acid and probably represent low-M_r proteoglycan and hyaluronic acid. The second major included peak contains the bulk of the cartilage proteins, and, in common with the third included peak, is devoid of uronic acid. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3*a*) of the column fractions revealed that the major peak (fractions 35–43, Fig. 2) contains prominent proteins of M_r 65000 (probably albumin) and 55000, together with the link proteins of M_r 48000, 44000 and 41000. The third included peak

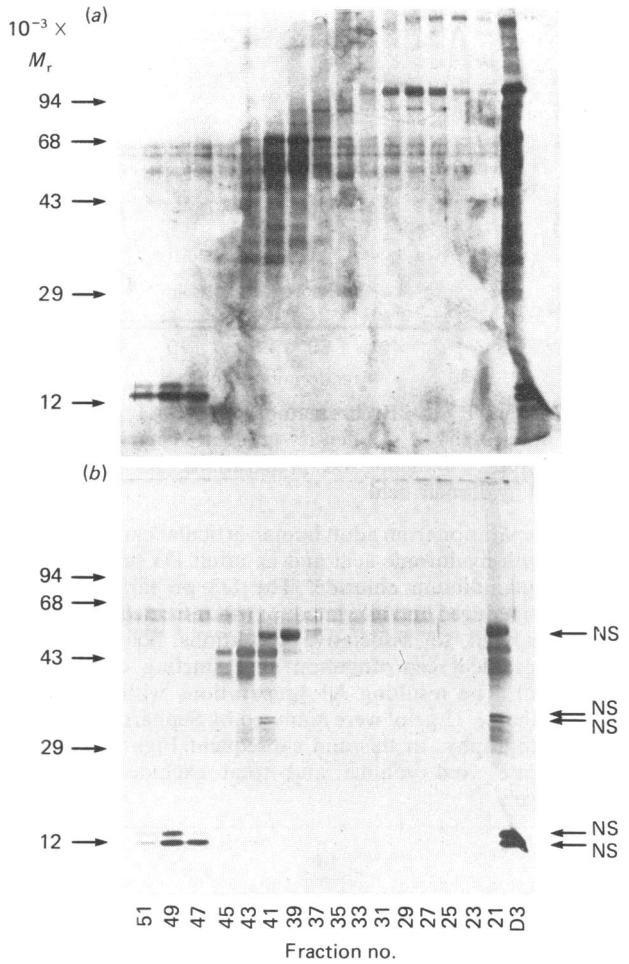

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions from Sepharose CL-6B chromatography of an adult D3 preparation

Fractions from the dissociative Sepharose CL-6B column of an adult D3 preparation (Fig. 2) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. Protein was detected (*a*) directly by staining with Coomassie Brilliant Blue, or (*b*) by immune localization with the use of an antiserum to the link proteins after transfer to nitrocellulose. The M_r values of reference proteins (phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c*) are indicated, and the pattern obtained with the unfractionated D3 preparation is also shown. NS indicates protein that stains non-specifically with the immune-localization procedure.

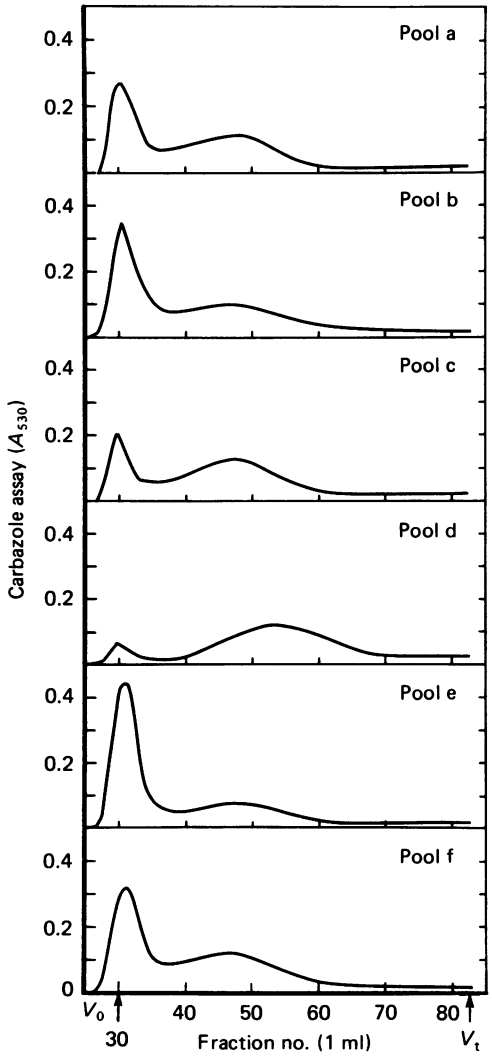

Fig. 4. Sepharose CL-2B chromatography of A1 preparations obtained by CsCl-density-gradient centrifugation of mixtures containing D1 preparations, fractions of a D3 preparation and hyaluronic acid

A D1 preparation from adult human articular cartilage was mixed with hyaluronic acid and fractions from an adult D3 preparation chromatographed through Sepharose CL-6B (a, b, c, d, e and f refer to the pools indicated in Fig. 2) in 4 M-guanidinium chloride. Samples were dialysed to associative conditions before CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

(fractions 45–51, Fig. 2) contains lysozyme, which was identified by the lysoplate assay method of Osserman & Lawler (1966).

It may be noted that, when immunoidentification is used to verify the position of the link protein, other proteins, of M_r about 55000, 35000 and 12000 (lysozyme), are also visible (Fig. 3*b*). These proteins are not specific reaction products as, unlike the link proteins, they are also apparent when non-immune sheep serum is used in the first step. It appears that they interact with the peroxidase coupled to the second-step antibody.

When fractions from the column were used in re-aggregation experiments in conjunction with the adult D1 preparation and hyaluronic acid (Fig. 4), the factor interfering with aggregate formation was found to reside in the major included peak (Fig. 4*d*; fractions 38–40, Fig. 2). It is of particular interest that neither the fractions containing the small proteoglycans (Figs. 4*a* and 4*b*) nor those containing the link proteins (Fig. 4*e*; fractions 41–43, Fig. 2) or lysozyme (Fig. 4*f*, fractions 44–50, Fig. 2) had any detrimental effect on the ability to form aggregates.

One possible explanation for these results is that the adult D3 preparation contains a protein that binds to hyaluronic acid in such a way that it prevents the binding

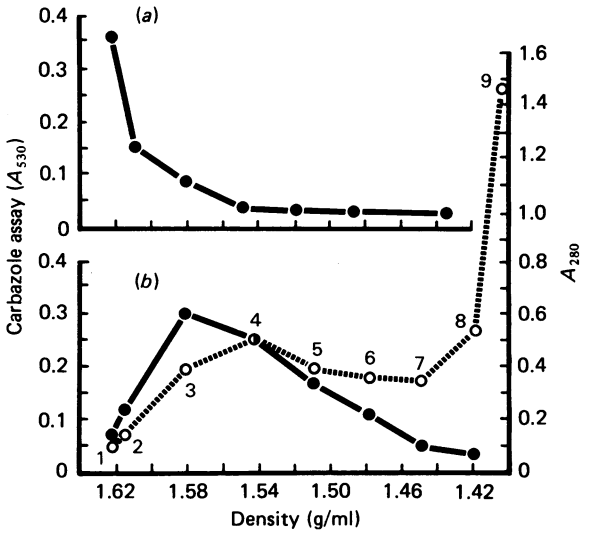

Fig. 5. CsCl-density-gradient centrifugation of hyaluronic acid in the absence and in the presence of a D3 preparation

Hyaluronic acid was subjected to CsCl-density-gradient centrifugation in (*a*) the absence or (*b*) the presence of an adult D3 preparation. The hyaluronic acid and the D3 preparation were mixed in 4 M-guanidinium chloride, then dialysed to associative conditions before centrifugation at a starting density of 1.50 g/ml. Fractions were collected and analysed for absorbance at 280 nm (○) and uronic acid content (●). Fractions (1–9) for further analysis are indicated.

of the proteoglycan subunits either by its greater avidity for binding or because of its presence in excess. In order to determine which proteins could bind, the adult D3 preparation and hyaluronic acid were mixed in 4 M-guanidinium chloride and dialysed to associative conditions before CsCl-density-gradient centrifugation with a starting density of 1.5 g/ml. To ensure complete interaction and maximal separation of a hyaluronic acid–protein complex from free protein, the hyaluronic acid and D3 preparation were mixed in a 1:4 weight ratio. In the absence of the D3 preparation the hyaluronic acid had a buoyant density close to 1.7 g/ml, whereas in the presence of the D3 preparation the buoyant density was decreased to near 1.58 g/ml (Fig. 5). Considerable protein was co-eluted with the hyaluronic acid, and on analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the only readily identifiable components were the link proteins (Fig. 6). None of the three other proteins previously mentioned in the D3 preparation (65000-M_r protein, 55000-M_r protein and lysozyme) appeared to bind under these conditions. Furthermore, the bound link proteins showed the fragmentation pattern characteristic of the adult (Mort *et al.*, 1983), indicating that such a modification does not prevent binding to hyaluronic acid. At this stage no obvious protein with a molecular size compatible with the factor responsible for interfering with proteoglycan aggregation was apparent on the gels, probably owing to the weak diffuse staining with Coomassie Blue exhibited by the factor (as indicated later in the text and in Fig. 12).

In order to achieve large-scale preparations of

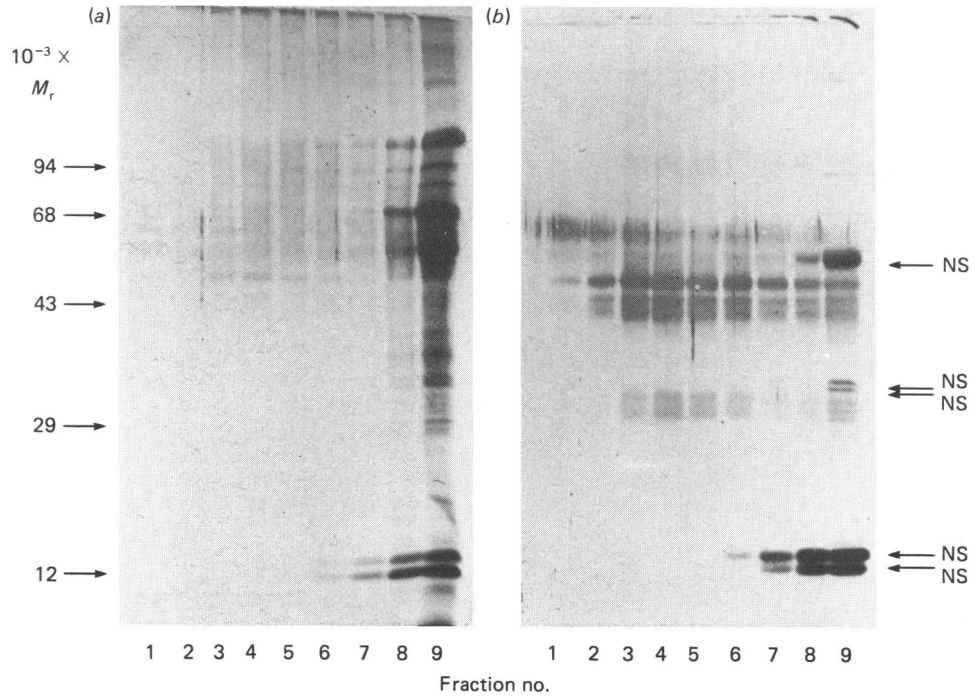

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions from the CsCl-density-gradient centrifugation of hyaluronic acid in the presence of a D3 preparation

Fractions from the CsCl-density-gradient centrifugation of hyaluronic acid in the presence of an adult D3 preparation (Fig. 5*b*) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. Protein was detected (*a*) directly by staining with Coomassie Brilliant Blue, or (*b*) by immune localization with the use of an antiserum to link proteins after transfer to nitrocellulose. M_r values of reference proteins are indicated, and NS refers to protein that stains non-specifically with the immune-localization procedure.

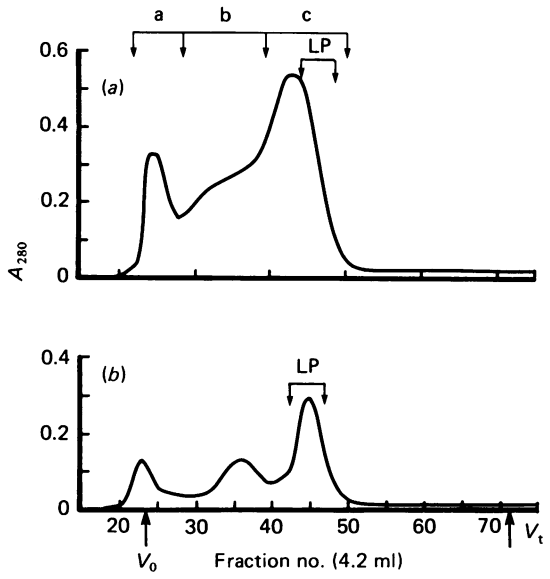

Fig. 7. Sepharose CL-6B chromatography of A1D3 preparations under dissociative conditions

A1D3 preparations were dissolved in 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5, and chromatographed in the same buffer through Sepharose CL-6B. The A1D3 preparations were from (*a*) adult cartilage and (*b*) neonatal cartilage. Fractions were collected and monitored for absorbance at 280 nm, then pooled (a, b and c) for further investigation. The elution position of link proteins (LP) is also shown.

hyaluronic acid-binding molecules, fresh cartilage was extracted and the extracts were dialysed to associative conditions, before the addition of CsCl to a density of 1.5 g/ml. An A1 preparation was then isolated, which was expected to contain proteoglycan subunits plus any hyaluronic acid–protein–proteoglycan complex. The hyaluronic acid-binding proteins were then separated from this mixture by a subsequent centrifugation under dissociative conditions as an A1D3 preparation. This preparation was subjected to chromatography on Sepharose CL-6B in the presence of 4 M-guanidinium chloride. The resulting profile (Fig. 7*a*) was similar to that obtained by the fractionation of the adult D3 preparation, except that the lysozyme peak was no longer present, and the major peak was devoid of the 65000-M_r protein and deficient in the 55000-M_r protein (Fig. 8).

It may be noted that when a neonatal A1D3 preparation was chromatographed under identical conditions a three-peak pattern was again obtained (Fig. 7 b). However, the major included peak was narrow compared with that in the adult fractionation, and on analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis appeared to contain only the link proteins (results not shown). The hyaluronic acid-binding protein of slightly larger size was therefore absent from the neonatal preparation.

The three protein peaks from the fractionation of the adult A1D3 preparation were checked for their ability to prevent the interaction of an adult D1 preparation with hyaluronic acid in re-aggregation experiments (Fig. 9). As expected, the component responsible for this property resided in the third (major included) peak (Fig. 9*c*). When a similar re-aggregation experiment was performed with

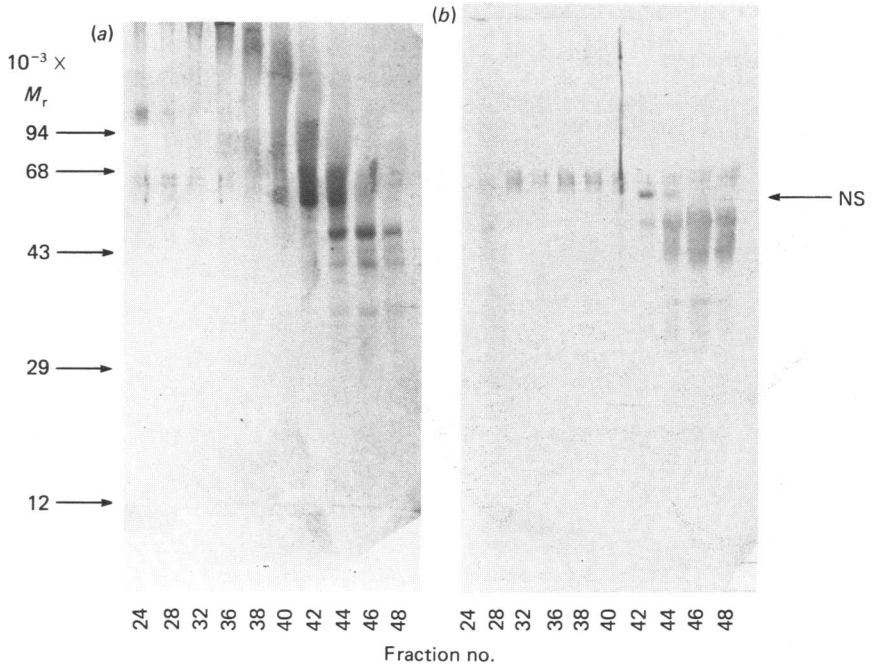

Fig. 8. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions from Sepharose CL-6B chromatography of an adult A1D3 preparation

Fractions from the dissociative Sepharose CL-6B column of an adult A1D3 preparation (Fig. 7) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. Protein was detected (*a*) directly by staining with Coomassie Brilliant Blue, or (*b*) by immune localization with the use of an antiserum to the link proteins after transfer to nitrocellulose. M_r values of reference proteins are indicated, and NS refers to protein that stains non-specifically with the immune-localization procedure.

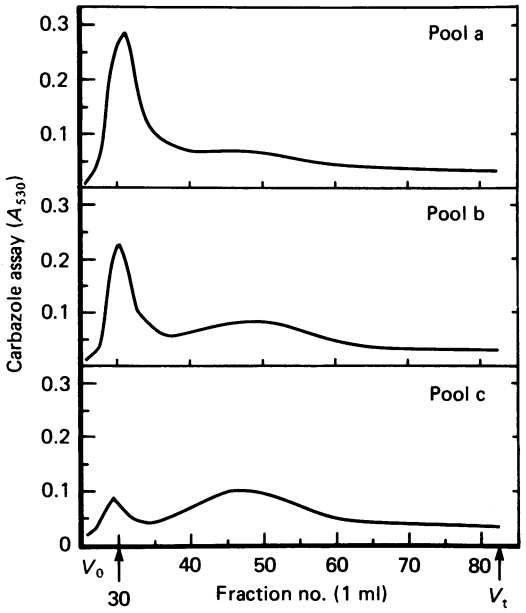

Fig. 9. Sepharose CL-2B chromatography of A1 preparations obtained by CsCl-density-gradient centrifugation of mixtures containing D1 preparations, fractions of an A1D3 preparation and hyaluronic acid

A D1 preparation from adult human articular cartilage was mixed with hyaluronic acid and fractions from an adult A1D3 preparation chromatographed through Sepharose CL-6B (a, b and c refer to the pools indicated in Fig. 7) in 4 M-guanidinium chloride. Samples were dialysed to associative conditions before CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

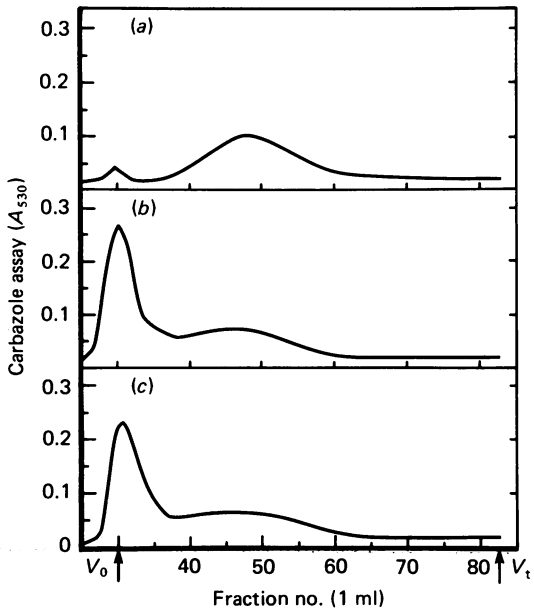

Fig. 10. Sepharose CL-2B chromatography of A1 preparations obtained by CsCl-density-gradient centrifugation of mixtures containing D1 preparations, hyaluronic acid and various cartilage protein preparations

A D1 preparation from adult human articular cartilage was mixed in 4 M-guanidinium chloride with hyaluronic acid and (*a*) an adult D3 preparation, (*b*) an adult A2 preparation, or (*c*) an adult A1D2 preparation. Samples were dialysed to associative conditions before CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml. The resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

an equal weight of an A2 preparation (proteoglycan and proteins not binding to hyaluronic acid) or an A1D2 preparation (low-density proteoglycans that bind to hyaluronic acid) replacing the A1D3 fraction, there was no interference with aggregation (Fig. 10). It thus would appear that, at the concentrations used, the only cartilage component capable of interfering with aggregation was a hyaluronic acid-binding protein with an apparent M_r of about 60000 on Sepharose CL-6B chromatography (relative to the elution positions of the proteins of M_r 65000 and 55000 previously identified in the D3 preparation).

In order to determine whether this component merely competes with the proteoglycan subunits for binding sites on hyaluronic acid or whether it can also displace the proteoglycan from hyaluronic acid when previously bound, a number of additional re-aggregation experiments were performed. In these the adult D3 preparation was mixed with (*a*) a neonatal D1 preparation and hyaluronic acid in 4 M-guanidinium chloride and then dialysed to associative conditions, (*b*) a mixture of neonatal D1 preparation and hyaluronic acid already under associative conditions (a proteoglycan–hyaluronic acid complex), and (*c*) a neonatal A1 preparation under associative conditions (a link-protein-stabilized proteoglycan aggregate). In the resulting A1 preparations obtained after centrifugation the first two conditions yielded mainly proteoglycan subunits, whereas the third condition yielded considerable aggregate (Fig. 11). Thus it would appear that the hyaluronic acid-binding protein can not only compete with the proteoglycan subunits for hyaluronic acid, but can also displace them even when they are previously bound, as long as the interaction is not link-protein-stabilized. As these results were obtained with neonatal proteoglycan subunits, it would appear that the protein is not restricted in its action to the adult human molecules.

It was of concern that this protein was not readily visible by conventional staining of the electrophoresis gels with Coomassie Blue. Several reasons are possible, such as the protein may be heterogeneous in size and so diffuse on the gel or it may be heavily glycosylated with oligosaccharides that interfere with dye binding. To attempt to resolve these points the adult D3 preparation and the fraction from the A1D3 preparation containing the protein in question were electrophoresed with the use of increased concentrations. In addition, a sample of purified hyaluronic acid-binding region, obtained by clostripain digestion of a neonatal proteoglycan aggregate (Roughley *et al.*, 1982), was used as a reference protein that may be expected to have similar properties. The purified hyaluronic acid-binding region migrated as a diffuse band, staining weakly with Coomassie Blue (Fig. 12). Under both reducing and non-reducing (results not shown) conditions the adult protein preparation showed diffuse staining in a similar position. When Stains All was used as the dye this diffuse area was the only discrete

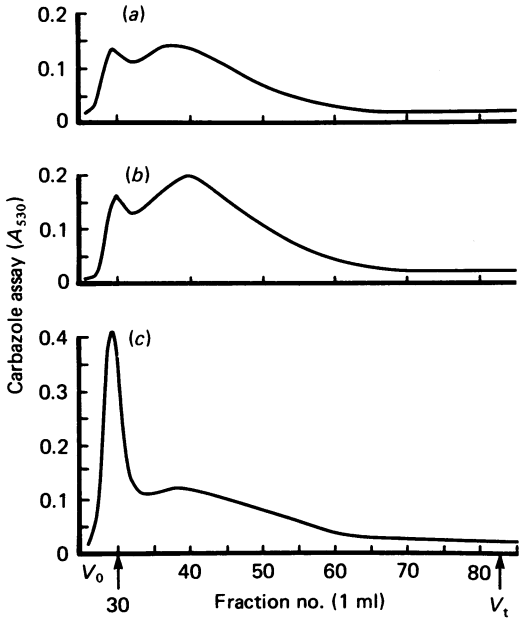

Fig. 11. Sepharose CL-2B chromatography of A1 preparations obtained by CsCl-density-gradient centrifugation of mixtures containing D3 preparations together with D1 preparations and hyaluronic acid or A1 preparations

A D3 preparation from adult human articular cartilage was mixed with (*a*) a neonatal D1 preparation and hyaluronic acid in 4 M-guanidinium chloride and then dialysed into 0.1 M-sodium acetate, (*b*) a neonatal D1 preparation and hyaluronic acid combination (proteoglycan–hyaluronic acid complex) in 0.1 M-sodium acetate, and (*c*) a neonatal A1 preparation (link-protein-stabilized proteoglycan aggregate) in 0.1 M-sodium acetate. Samples were subjected to CsCl-density-gradient centrifugation at a starting density of 1.68 g/ml, and the resulting A1 preparations with densities greater than 1.72 g/ml were analysed by Sepharose CL-2B chromatography.

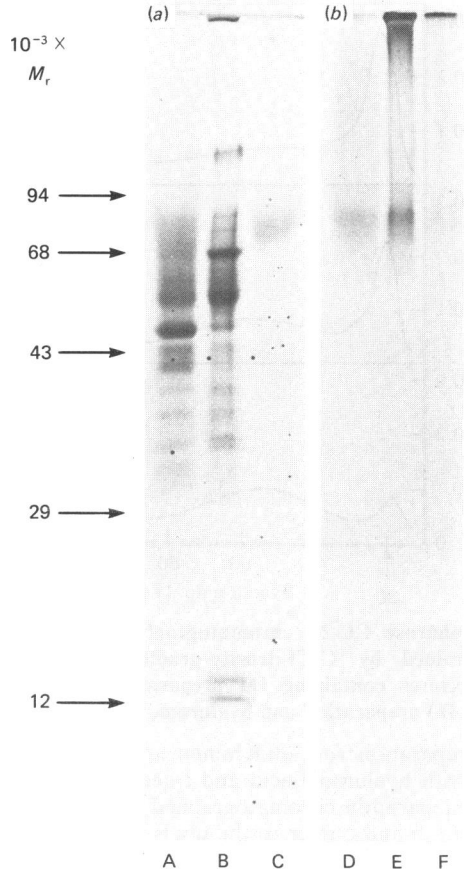

Fig. 12. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of various cartilage protein preparations

An adult A1D3 fraction (A and D), an adult D3 preparation (B and E) and a neonatal hyaluronic acid-binding region preparation (C and F) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. Protein was detected by staining with (*a*) Coomassie Brilliant Blue (A, B and C) or (*b*) Stains All (D, E and F). The A1D3 fraction was pool c (Fig. 7) and the hyaluronic acid-binding region was prepared by clostripain digestion of a proteoglycan aggregate preparation (Roughley *et al.*, 1982).

staining within the gel. It thus would appear that the hyaluronic acid-binding protein of adult human cartilage that interferes with the preparation of high-density proteoglycan aggregates is similar in its size and staining characteristics to an isolated hyaluronic acid-binding region derived from an intact proteoglycan. By this technique the hyaluronic acid-binding protein appears to have an M_r of about 75000.

In an attempt to confirm the identity of the protein the fractions from the chromatography of an adult A1D3 preparation were analysed by radioimmunoassay for their content of keratan sulphate and hyaluronic acid-binding region (Fig. 13). Keratan sulphate was predominant at the void volume and in the first included peak, whereas hyaluronic acid-binding region was also present in the other included peak, which is the site of the hyaluronic acid-binding protein under investigation. This would be compatible with the larger species being small proteoglycan subunits and the protein being the hyaluronic acid-binding region derived from such subunits. The relatively low degree of immunoreactivity with the anti-(hyaluronic acid-binding region) antibody may indicate a conformational change has taken place upon generation of the protein from more intact proteoglycan subunits.

DISCUSSION

This paper describes the occurrence of a protein, of M_r about 60000–75000 (depending on the technique used), that is present in normal adult human articular cartilage but not neonatal cartilage, and that through its ability to bind to hyaluronic acid can prevent the isolation of high-density proteoglycan aggregates. The protein can not only compete with the proteoglycan subunits for binding sites on the hyaluronic acid, but can also displace the proteoglycan subunit from the hyaluronic acid once it is already bound, if the binding is not stabilized by the further interaction of link proteins. This protein resembles a hyaluronic acid-binding region of a proteoglycan subunit obtained by proteolytic treatment of a proteoglycan aggregate in both its size and functional properties. This is in fact its likely origin, as the protein does show immunological cross-reactivity with an antiserum to such a binding region derived from the Swarm rat chondrosarcoma. If this is the case then

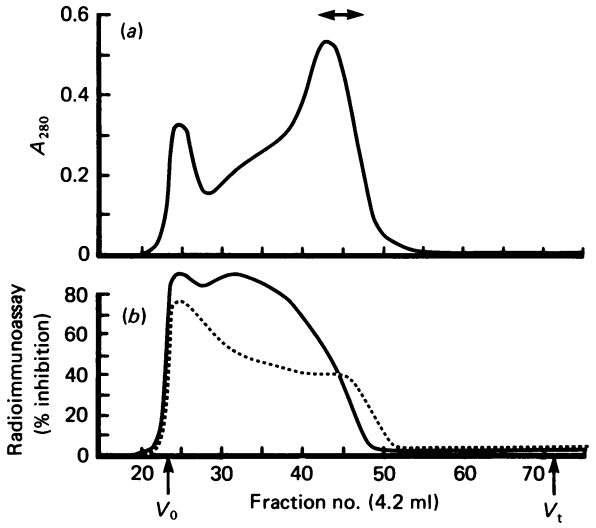

Fig. 13. Radioimmunoassay of fractions from Sepharose CL-6B chromatography of an adult A1D3 preparation

Fractions from a dissociative Sepharose CL-6B column of an adult A1D3 preparation were analysed for (*a*) absorption at 280 nm and (*b*) immunoreactivity towards antisera directed against keratan sulphate (——) or a hyaluronic acid-binding region of a proteoglycan subunit (·····). The position of the material that interferes with the preparation of high-buoyant-density proteoglycan aggregates is indicated (←→).

proteolytic cleavage would appear to have proceeded to such an extent that all detectable glycosaminoglycan chains have been removed. The presence of a similar protein with M_r in the range 50000–70000 has also been alluded to in adult human costal cartilage (Pearson & Mason, 1979).

It is noteworthy that other low-density molecules present in the tissue also contain a functional hyaluronic acid-binding region. These molecules probably represent partially cleaved proteoglycan subunits; they do not, however, interfere with the formation of the high-density proteoglycan aggregates in a manner analogous to that of the presumptive hyaluronic acid-binding region. One might therefore speculate that the hyaluronic acid-binding region free of glycosaminoglycan chains might have a greater avidity for hyaluronic acid than its counterpart that is part of a proteoglycan. This may be due to a change in conformation of the hyaluronic acid-binding region or a diminution in steric hindrance upon removal of the glycosaminoglycan-attachment region.

Two other possibilities for the interference in aggregation were shown to be negative in this tissue. Firstly, the link protein, which appears partially fragmented in the adult human (Mort *et al.* 1983), did not appear to affect binding of the proteoglycan subunits to hyaluronic acid. One cannot, however, comment on the stability of such a link-protein-stabilized interaction towards displacement by reagents such as oligosaccharides derived from hyaluronic acid. Secondly, the lysozyme present in the adult cartilage did not interfere with the formation of stable proteoglycan aggregates. Others have reported that lysozyme can disaggregate cartilage proteoglycans (Kuettner *et al.*, 1974), though Greenwald (1976) found no such effect.

Finally, it is worthwhile considering what these observations mean in relation to the structure of the cartilage matrix. One might envisage that, during maturation of the cartilage in the adult, turnover of matrix macromolecules via proteolysis is proceeding continuously at a low rate. Over a long period of time this will result in a build-up of hyaluronic acid-binding regions linked to the hyaluronic acid network within the tissue, especially if replacement of these molecules by newly made intact proteoglycans is also slow. This would be compatible with the increased content of non-collagenous protein reported to occur during aging (Muir, 1970; Venn, 1978). As a result, many hyaluronic acid-binding sites that could be occupied by intact proteoglycans are not available, and this would be expected to decrease the fixed glycosaminoglycan content (charge density) of the cartilage, and thereby may impair the ability of the tissue to resist compressive loading. One would predict that those persons in whom this degradative process was most pronounced may be more susceptible to the pathological changes characteristic of osteoarthrosis. However, one should also point out that the hyaluronic acid concentration of human articular cartilage has been shown to increase with age (Pearson & Mason, 1979; Elliott & Gardner, 1979), and this may act as a compensatory mechanism in normal individuals, as it may produce additional sites for the binding of proteoglycan subunits.

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