Hyaluronic Acid in Cartilage and Proteoglycan Aggregation

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1. Dissociation of purified proteoglycan aggregates was shown to release an interacting component of buoyant density higher than that of the glycoprotein-link fraction of Hascall & Sajdera (1969). 2. This component, which produced an increase in hydrodynamic size of proteoglycans on gel chromatography, was isolated by ECTEOLA-cellulose ion-exchange chromatography and identified as hyaluronic acid. 3. The effect of pH of extraction showed that the proportion of proteoglycan aggregates isolated from cartilage was greatest at pH4.5. 4. The proportion of proteoglycans able to interact with hyaluronic acid decreased when extracted above or below pH4.5, whereas the amount of hyaluronic acid extracted appeared constant from pH3.0 to 8.5. 5. Sequential extraction of cartilage with 0.15M-NaCl at neutral pH followed by 4M-guanidinium chloride at pH4.5 was shown to yield predominantly non-aggregated and aggregated proteoglycans respectively. 6. Most of the hyaluronic acid in cartilage, representing about 0.7% of the total uronic acid, was associated with proteoglycan aggregates. 7. The non-aggregated proteoglycans were unable to interact with hyaluronic acid and were of smaller size, lower protein content and lower keratan sulphate content than the disaggregated proteoglycans. Together with differences in amino acid composition this suggested that each type of proteoglycan contained different protein cores.

Proteoglycans of cartilage are complex heteropolysaccharides in which a large number of chondroitin sulphate and keratan sulphate chains are linked to a polypeptide backbone. A large proportion can be extracted with 4M-guanidinium chloride and have been shown to contain both aggregated and non-aggregated molecules (Sajdera & Hascall, 1969; Rosenberg *et al.*, 1970; Tsiganos *et al.*, 1971; Mashburn & Hoffman, 1971). Equilibrium density-gradient centrifugation of the purified proteoglycans from bovine nasal septum in the presence of 4M-guanidinium chloride was shown to dissociate aggregates and separate a protein-rich fraction from the majority of the proteoglycans (Hascall & Sajdera, 1969). This fraction was found to be necessary for the reassociation of proteoglycans into aggregates and was therefore termed 'glycoprotein-link'. A similar fractionation was applied to proteoglycans from pig laryngeal cartilage (Tsiganos *et al.*, 1971), but the protein-rich fraction, although binding to proteoglycans, did not promote aggregation (Tsiganos *et al.*, 1972). However, a fraction from the gradient, of density (1.45-1.57g/ml) intermediate between the protein-rich component and the proteoglycan, was found to interact with the proteoglycan, producing an increase in its apparent size on gel chromatography (Tsiganos *et al.*, 1972). This effect was also produced by comparable fractions of proteoglycans from various types of cartilage, including bovine nasal septum. This

suggested that there was a component in the protein-rich fraction of Hascall & Sajdera (1969) of higher density that could be separated under the conditions used by Tsiganos *et al.* (1971). The increase in size of proteoglycans shown by gel chromatography was not accompanied by an increase in rate of sedimentation in the ultracentrifuge (R. Pain, personal communication). The effect thus did not constitute the complete re-formation of proteoglycan aggregates as described by Hascall & Sajdera (1969), which was characterized by a large increase in sedimentation coefficient. The isolation and characterization of the component of higher buoyant density and its distribution in aggregated and non-aggregated proteoglycan fractions is described here, and its interaction with proteoglycans examined. A preliminary report of part of this work has been published (Hardingham & Muir, 1973a).

Materials

All reagents were of analytical grade except for galactosamine hydrochloride, glucosamine hydrochloride, glucuronolactone, carbazole, guanidinium chloride and acetylacetone. The guanidinium chloride was purified with activated charcoal (Norit N.K., Hopkin and Williams, Chadwell Heath, Essex, U.K.) and acetylacetone was redistilled (b.p. 133-134°C).

Cetylpyridinium chloride, special grade (lot 30601), was obtained from AB Kabi, Stockholm,

Sweden. Chondroitin 4-sulphate was prepared from pig laryngeal cartilage as described by Hardingham & Muir (1970). Hyaluronic acid (from umbilical cord) and twice-crystallized papain (4units/mg of protein) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Crude papain (type II) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and testicular hyaluronidase (1000units/mg) from Boehringer, Mannheim, W. Germany. Gelatin (Bacto) was obtained from Difco Laboratories, Detroit, Mich., U.S.A., and Visking tubing, width 24/32 in and 8/32 in, for dialysis from Scientific Instruments Centre, London W.C.1, U.K.

Methods

Analytical methods

Uronic acid was determined by an automated modification (Heinegård, 1973) of the method of Bitter & Muir (1962) with glucuronolactone as standard. Hexosamine was determined by the procedure of Kraan & Muir (1957) with galactosamine hydrochloride as standard. Samples were hydrolysed in 8M-HCl for 3 h at 95°C (Swann & Balazs, 1966) in glass tubes sealed under N₂. Excess of acid was removed by rotary evaporation at 40°C and the hydrolysate was adjusted to a known volume with water. Glucosamine/galactosamine molar ratios were determined in the same hydrolysate by using the short column of a Locarte automated amino acid analyser. Sulphate was determined after hydrolysis of the sample in 60% (v/v) formic acid for 8 h at 100°C. Acid was removed by rotary evaporation and sulphate determined by the method of Dodgson (1961) after the addition of a known volume of water. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as standard and values were generally about 20–30% higher than the protein estimated by summation of amino acid analyses. Hydroxyproline was measured by the method of Woessner (1961) after hydrolysis in 6M-HCl at 105°C for 24 h in screw-top Teflon-lined glass tubes (Kimax, A. R. Horwell Ltd., London N.W.6, U.K.) Excess of acid was removed by rotary evaporation and the sample was dissolved in a known volume of water for analysis. Amino acid analyses were performed on a Locarte amino acid analyser by using a three-buffer single-column elution system. Samples containing 200–300 µg of protein in 2 ml of 6M-HCl were hydrolysed at 105°C for 24 h in glass tubes sealed under N₂. Excess of acid was removed by rotary evaporation at 40°C and the residue was dissolved in 1.0 ml of water and a sample (0.5 ml) was applied to the column of the analyser. No corrections

were made for the destruction of amino acids during hydrolysis.

Preparative methods

Extraction of proteoglycans with 4M-guanidinium chloride. Pig laryngeal cartilage was obtained fresh from the slaughterhouse, dissected free of adhering tissue and perichondrium and finely shaved with a Stanley Surform (woodwork tool). The cartilage was suspended in 10 times its weight of cold 4M-guanidinium chloride buffered at pH 5.8 or at pH 4.5 with 0.05M-sodium acetate and was agitated for 24 h at 4°C. The extract was filtered through two layers of lint on a Buchner funnel and the cartilage washed with a small volume of the buffered 4M-guanidinium chloride. The combined extract and washings were dialysed against 7 vol. of 0.05M-sodium acetate, pH 5.8. The proteoglycans were then purified by equilibrium density-gradient centrifugation as described below.

In separate experiments to examine the effect of pH, proteoglycans were extracted from cartilage by the same procedure with the following changes. The 4M-guanidinium chloride was buffered to pH 3.0, 4.5, 5.8 and 7.0 with sodium citrate-phosphate buffer (McIlvaine, 1921) used at 25% of the recommended strength and to pH 8.5 with 0.05M-Tris-HCl. After extraction the combined extracts and washings were adjusted to pH 5.8 with acetic acid or with 4M-NaOH as necessary and then dialysed against 7 vol. of 0.05M-sodium acetate, pH 5.8. The proteoglycans were purified as described below.

Sequential extraction of proteoglycans with 0.15M-NaCl followed by 4M-guanidinium chloride. Sliced cartilage prepared as described above was suspended in 10 times its weight of 0.15M-NaCl and agitated for 3 h at 4°C. Although unbuffered the pH remained at about pH 7. The extract was filtered on a coarse glass sinter (grade 1) and the proteoglycans were precipitated by the dropwise addition of 10% (w/v) cetylpyridinium chloride. The precipitate was centrifuged and washed twice with 0.05% (w/v) cetylpyridinium chloride containing 5 mM-Na₂SO₄ and once with 0.05% (w/v) cetylpyridinium chloride alone. The precipitate was dissolved in a minimum volume of propan-1-ol and re-precipitated as the sodium salt after the addition of a few drops of saturated sodium acetate and 5 vol. of ethanol. The precipitate was left overnight at 4°C and then centrifuged, washed with 80% (v/v) ethanol, then with ethanol and dried *in vacuo*.

The cartilage residue from the extraction with 0.15M-NaCl was then suspended in 10 times its weight of 4M-guanidinium chloride–0.05M-sodium acetate, pH 4.5, and extracted for 24 h at 4°C. The proteoglycans were isolated as described above for

the single-step extraction with 4M-guanidinium chloride.

Purification of proteoglycans by equilibrium density-gradient centrifugation under 'associative' conditions. The dialysed 4M-guanidinium chloride extract containing about 5mg of proteoglycan/ml was adjusted to a density of 1.60g/ml by the addition of solid CsCl. Equilibrium density-gradient centrifugation was performed in an MSE 65 centrifuge in an 8×25ml angle head at 95000g_{av} for 48h at 20°C. The tubes were then quickly frozen in a solid-CO₂-acetone bath and cut into two fractions. The bottom fraction (5ml), containing a thick proteoglycan gel, accounted for 91–96% of the total uronic acid. It was separated from the upper fraction (13ml), which contained collagen and other protein-rich components. The proteoglycan gel was dispersed by using a Pasteur pipette and was then either used directly for dissociative density-gradient centrifugation or dialysed to remove CsCl and used as the starting material for other experiments.

Fractionation of proteoglycans by equilibrium density-gradient centrifugation under 'dissociative' conditions. (1) To the bottom fraction of purified proteoglycans, prepared as described above, was added an equal volume of 7.5M-guanidinium chloride–0.05M-sodium acetate, pH5.8, and the density adjusted to 1.50g/ml by the addition of solid CsCl. Sufficient 4M-guanidinium chloride–CsCl solution of density 1.50g/ml was then added to dilute the proteoglycan concentration to 4–6mg/ml. The solution was then centrifuged under the same conditions as described above. After centrifugation the tubes were quickly frozen in a solid CO₂-acetone bath and cut into three fractions, i.e. bottom, 4ml, middle, 10ml, and top, 4ml. Samples of the fractions were either used directly for experiments or the fractions were dialysed against 0.5M-sodium acetate, pH6.8, for subsequent analysis.

(2) The proteoglycans extracted with 0.15M-NaCl were dissolved overnight in 4M-guanidinium chloride–0.05M-sodium acetate, pH5.8, adjusted to a density of 1.50g/ml with solid CsCl and then centrifuged and fractionated as described above.

When isolating the three fractions from the dissociative gradient, it was found that by freezing and cutting the tubes there was less cross-contamination of the fractions than when the tubes were pierced and drained from the bottom, or when a dense inert fluorocarbon liquid was pumped into the bottom of the pierced tube and fractions were collected from the top. It was impractical to cut the frozen tubes into a large number of fractions, however, so when the distribution of material through the gradient was examined in more detail, the tubes were pierced at the bottom and the liquid was slowly drained. Twelve fractions (each 1.5ml) were collected.

Recombination of fractions separated by 'dis-

sociative' density-gradient centrifugation. Samples of undialysed fractions were mixed in the same relative proportions in which they occurred in the gradient and were then dialysed overnight against a large volume of 0.5M-sodium acetate, pH6.8. Alternatively, before mixing, each fraction was dialysed separately against 4M-guanidinium chloride to remove CsCl and then mixed with the other component(s) and dialysed against 0.5M-sodium acetate, pH6.8. Both methods gave the same results.

The interacting component was assayed as follows. Samples to be tested were adjusted to 4M-guanidinium chloride by adding concentrated guanidinium chloride solution containing 0.05M-sodium acetate, pH5.8. They were then mixed with an excess of disaggregated proteoglycan in 4M-guanidinium chloride–0.05M-sodium acetate, pH 5.8, and dialysed as described above. The samples were then examined by gel chromatography on Sepharose 2B. Since disaggregated proteoglycan contained very little material that was eluted in the region of the void volume of the column, the increase in material eluted in this region was taken as a measure of the extent of interaction. This was estimated by cutting and weighing a tracing of the uronic acid elution profile.

Preparation of glycosaminoglycans. Samples of freshly sliced cartilage or the washed cartilage residue after 4M-guanidinium chloride extraction were suspended in 3–5 times their weight of 0.1M-sodium acetate, pH5.5, containing 0.05M-EDTA (disodium salt) and 0.01M-L-cysteine. Activated (Kimmel & Smith, 1954) crude papain (20mg/g wet wt. of cartilage) or crystalline papain (0.5mg/g wet wt. of cartilage) was added to the suspension, which was incubated at 60°C for 4h. The insoluble residue was discarded. After dilution of the digest with an equal volume of water, the glycosaminoglycans were precipitated by the dropwise addition of 10% (w/v) cetylpyridinium chloride. The precipitate was isolated and converted into the sodium salt, washed and dried as described for the proteoglycan extracted with 0.15M-NaCl.

Chromatographic techniques

ECTEOLA-cellulose ion-exchange chromatography. ECTEOLA-cellulose (Serva, Heidelberg, Germany) was washed with 4M-HCl followed by water until the washings reached pH5. Fines and dissolved air were then removed and the cellulose was packed in a column (16.0cm×1.1cm). Samples of the middle fraction from 'dissociative' density-gradient centrifugation containing 1.0–10.0mg of uronic acid were dialysed exhaustively against water and then conc. HCl was added to a final concentration of 0.02M. Samples were applied to the column, washed in with 20ml of 0.02M-HCl and then eluted with 20ml each of 0.5M-NaCl, 2.5M-NaCl and 4M-HCl. The acidic

fraction was neutralized with NaOH as soon as it emerged from the column. The last two fractions were dialysed against a large excess of 0.5M-sodium acetate, pH6.8. After concentration by pressure dialysis by using an Amicon Diaflo apparatus with a UM-10 membrane the uronic acid and hexosamine contents and galactosamine/glucosamine molar ratios of each fraction were determined, and the interaction with proteoglycan was tested as described above. Fractions containing more than 1mg of the interacting component were concentrated and the material was then precipitated at 4°C by the addition of 5vol. of ethanol. The precipitates were centrifuged, washed with ethanol and dried *in vacuo*.

Gel chromatography. (1) Samples of proteoglycan containing about 1mg of uronic acid/ml in 0.5M-sodium acetate, pH6.8, were applied to a column (165cm×1.1cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) which was eluted upwards with 0.5M-sodium acetate, pH6.8, at 4°C at the rate of 6ml/h by using a peristaltic pump. Fractions (about 2.5ml; 40 drops) were collected and their uronic acid and/or protein contents determined. Proteoglycan aggregates and glucuronolactone were used as markers of the void volume and total column volume respectively.

(2) A sample of the middle fraction from the dissociative density gradient was chromatographed on a column (90cm×0.9cm) of Sepharose 2B, eluted with 4M-guanidinium chloride, pH5.8. Nine fractions of equal volume were collected from the region of the void volume (V_0) of the column to the total volume (V_t) of the column. The ability of the fractions to interact with proteoglycans was tested by gel chromatography as described above, and their uronic acid contents were determined after dialysis against several volumes of 0.5M-sodium acetate, pH6.8.

(3) Samples of the purified interacting component containing about 100μg of uronic acid were applied to a column (120cm×0.9cm) of Sephadex G-200 eluted with 0.2M-sodium acetate, pH6.8, by using a hydrostatic head of 60cm. Fractions (2ml) were collected and pooled into nine equal fractions from the void volume to the total volume of the column.

The ability of these fractions to interact with proteoglycan was tested as described above.

Experiments on the interacting component

Hyaluronidase digestion. A sample of the purified interacting component was incubated overnight at 37°C in 0.1M-sodium acetate buffer, pH5.3, containing 100μg of testicular hyaluronidase/ml. Boiled enzyme was added in place of active enzyme in a control incubation. The ability of the digested and control samples to interact with disaggregated proteoglycans was tested as described above.

Papain digestion. A sample of the purified fraction was incubated with activated crystalline papain as described for the digestion of cartilage. At the end of the incubation it was placed in a boiling-water bath for 5min and a drop of saturated CaCl₂ added. It was then cooled and chromatographed directly on a column of Sephadex G-200 as described above.

Alkali treatment. To a sample of the purified fraction in 0.5M-NaCl was added an equal volume of 1.0M-NaOH. The solution was gassed with N₂ for 5min, stoppered and left at 4°C overnight. The solution was then neutralized with the calculated volume of acetic acid and chromatographed on a column of Sephadex G-200 as described above.

Infrared spectroscopy. A portion (1.0mg) of the purified interacting component from ECTOLA-cellulose ion-exchange chromatography and 100mg of KCl were dissolved in 2ml of water and the solution was freeze-dried. The resulting powder was compressed into a disc and the i.r. spectrum recorded on a Unicam SP.200 spectrophotometer. Spectra were also obtained from samples of hyaluronic acid from umbilical cord and chondroitin 4-sulphate from pig laryngeal cartilage by the same procedure.

Results and Discussion

Identification of the interacting component

Proteoglycans extracted from pig laryngeal cartilage with 4M-guanidinium chloride, pH5.8, were purified by equilibrium density-gradient centrifugation under 'associative' conditions. They were

Table 1. Analysis of fractions obtained by equilibrium density-gradient centrifugation of proteoglycans, under dissociative conditions

For conditions see the text.

Fraction	Density (g/ml)	Volume (ml)	Contents (% of total)		Galactosamine/ glucosamine molar ratio
			Uronic acid	Protein (Folin)	
Top	<1.43	4	2.3	31.8	2.2
Middle	1.43-1.57	10	5.0	7.2	4.3
Bottom	>1.57	4	92.7	61.0	12.6

then separated into three fractions by a second density gradient under 'dissociative' conditions in the presence of 4 M-guanidinium chloride at pH 5.8. The fraction of highest density (>1.57 g/ml) contained most of the proteoglycan, and a fraction rich in protein separated at the top of the gradient (<1.43 g/ml). A fraction that accounted for only 5% of the total uronic acid and 7.2% of the total protein was obtained from the middle of the gradient (1.43–1.57 g/ml). Analyses of the three fractions are given in Table 1.

The effect of combining samples from the top and the middle of the gradient on the hydrodynamic size of the proteoglycan from the bottom of the gradient was assessed by gel chromatography on Sepharose 2B (Figs. 1a–1d). The samples were mixed

Fig. 1. Gel chromatography on Sepharose 2B of proteoglycan fractions after disaggregation

Proteoglycans were fractionated in a dissociative density gradient as shown in Table 1. Fractions were recombined in the same proportions in which they occurred in the gradient, dialysed against 0.5 M-sodium acetate, pH 6.8, and samples applied to a column (165 cm $\times$ 1.1 cm) of Sepharose 2B as described in the text. Fractions were analysed for uronic acid (—) and protein (----). (a) Disaggregated proteoglycan (bottom fraction); (b) disaggregated proteoglycan + middle fraction; (c) disaggregated proteoglycan + top fraction; (d) disaggregated proteoglycan + middle and top fractions.

in the same relative proportions in which they occurred in the gradient.

The fraction from the middle of the gradient increased the hydrodynamic size of about 50% of the proteoglycans (Fig. 1b), whereas the protein-rich fraction from the top of the gradient changed the elution of the proteoglycan very little, even though protein became bound to the proteoglycan and was eluted with it (Fig. 1c) (Tsiganos *et al.*, 1972). Combining all three density-gradient fractions did not enhance the effect (Fig. 1d), implying that the active component was present exclusively in the middle fraction. There was a linear relationship between the amount of the middle fraction added to the proteoglycan and the percentage of the total uronic acid excluded by gel chromatography on Sepharose 2B (Fig. 2), up to a proportion of middle fraction to proteoglycan of 0.75:1 (w/v) (1:1 corresponded to the proportion in which they occurred in the gradient). When this proportion was increased no more proteoglycan was excluded from the gel, and the amount remained constant at 52%, suggesting that 48% was unable to interact. The middle fraction thus contained more activity than was required to produce the maximum effect on the proteoglycan, although subsequent experiments showed that with optimal conditions of preparation a larger proportion of proteoglycans was able to interact. The results showed that the interacting component could be assayed by mixing with disaggregated proteoglycans from the bottom fraction of the density

Fig. 2. Interaction between disaggregated proteoglycans and the middle fraction from the dissociative gradient

Various proportions of the middle fraction were mixed with a constant amount of disaggregated proteoglycan (bottom fraction). Samples were chromatographed on a column (165 cm $\times$ 1.1 cm) of Sepharose 2B and the proportion of uronic acid excluded from the gel was determined as described in the text. A ratio of middle fraction to proteoglycan of 1.0 corresponded to the proportion in which they occurred in the gradient.

gradient and measuring the change in elution profile by gel chromatography. When the relative proportion of interacting component to proteoglycan was below that required to give the maximum effect the method could be used to determine quantitatively the amount of activity.

From its analysis (Table 1) and behaviour on gel chromatography (Tsiganos *et al.*, 1971) the middle density-gradient fraction contained proteoglycans of higher protein content than those in the bottom fraction. Attempts were thus made to subfractionate this material to discern whether ability to interact with proteoglycans was a property of the material as a whole or of a minor constituent. It seemed likely that if it were a minor constituent, it would be bound to the proteoglycans at low ionic strength and dissociated at high ionic strength. An attempt was therefore made to separate the active component from proteoglycans (containing uronic acid) by gel chromatography on Sepharose 2B in the presence of 4M-guanidinium chloride, pH 5.8, but the proteoglycans were eluted as a broad retarded peak, which largely coincided with the activity (Fig. 3). It was evident, however, that the activity was not proportional to the uronic acid content of the fractions. Thus although the fractionation failed to separate activity from proteoglycan it suggested that the former was not a property of the latter.

Ion-exchange chromatography on ECTEOLA-cellulose, however, successfully separated the activity from the proteoglycan, since only 10% of the uronic acid was eluted in the 0.5M-NaCl fraction, which contained 73.2% of the activity. Analysis of this fraction showed that it contained equimolar amounts of uronic acid and hexosamine and had a glucosamine/galactosamine molar ratio of 25:1 (Table 2). The fractions eluted later accounted for 83% of the total uronic acid and from the composition appeared to contain proteoglycans with relatively high protein and glucosamine contents (Table 2)

compared with proteoglycans in the bottom fraction of the density gradient (Table 1).

The carbohydrate composition of the active fraction isolated by ECTEOLA-cellulose ion-exchange chromatography suggested that it was a glycosaminoglycan, which, since it was mostly eluted in the void volume on Sephadex G-200, was large. Moreover, the position of elution was unaltered after treatment with papain or exposure to alkaline conditions that induce β -elimination of

Fig. 3. Gel chromatography on Sepharose 2B in 4M-guanidinium chloride of the middle fraction from the dissociative gradient

A sample of the middle fraction containing about 1 mg of uronic acid was applied to a column (90cm×0.9cm) of Sepharose 2B eluted with 4M-guanidinium chloride-0.05M-sodium acetate, pH 5.8. Nine fractions of equal volume from the region of the void volume (V_0) to the total volume (V_t) were collected. One-tenth of each fraction was mixed with disaggregated proteoglycan (containing 1mg of uronic acid) and the interaction measured by gel chromatography as described in the text (■). After dialysis against 0.5M-sodium acetate, pH 6.8, the uronic acid content of each of the nine fractions was determined (●).

Table 2. Analysis of fractions obtained by ECTEOLA-cellulose ion-exchange chromatography of the material from the middle (density 1.43–1.56g/ml) of the dissociative density gradient (as shown in Table 1)

A sample containing about 1 mg of uronic acid was applied to a column (16.0cm×1.1 cm) of ECTEOLA-cellulose and eluted as described in the text. Acid fractions were neutralized immediately they were eluted from the column and pooled fractions from each eluate were concentrated by pressure dialysis (Diaflo) before analysis.

Eluent fraction	Uronic acid content (μg)	Protein (Folin) content (μg)	Molar ratios		Interaction with proteoglycan (% of recovered activity)
			Sulphate uronic acid	Glucosamine galactosamine	
0.02M-HCl	40	140	—	—	1.0
0.5M-NaCl	78	50	0.1*	25.0	73.2
2.5M-NaCl	244	670	1.06	0.16	{ 10.0 16.8
4M-HCl	467	2120			

* Analysis on comparable fraction from larger sample.

polysaccharide chains linked to hydroxyamino acids. Thus the activity of the component was not dependent on an integral protein moiety. On the other hand, the ability of the component to interact with the proteoglycans was completely abolished by digestion with testicular hyaluronidase. These properties together with the analysis suggested that the active component was hyaluronic acid. This was confirmed by the i.r.-absorption spectrum, which showed no absorption at 1240cm^{-1} characteristic of sulphate groups and which resembled the spectrum of authentic hyaluronic acid (Fig. 4).

A sample of the interacting component containing 0.5mg of uronic acid was fractionated by gel chromatography on a column of Sepharose 2B

(Fig. 5a) and showed a broad distribution of size comparable with that observed in the presence of 4 M-guanidinium chloride (Fig. 3). The eluted material was arbitrarily divided into three fractions and each tested separately with proteoglycan (Fig. 5b). The activity of each fraction was directly proportional to the amount of uronic acid in it and not to the position of elution, which further suggested that the active component was hyaluronic acid, and not some other compound. Moreover, hyaluronic acid from other sources, such as umbilical cord and cock's comb, also interacted in a similar way with cartilage proteoglycans (Hardingham & Muir, 1972b).

The results showed that during the fractionation of proteoglycan aggregates in a dissociative density

Fig. 4. Infrared absorption spectra

I.r. spectra of (a) ECTEOLA-cellulose 0.5 M-NaCl fraction; (b) hyaluronic acid from human umbilical cord (BDH Chemicals Ltd.); (c) chondroitin 4-sulphate from pig laryngeal cartilage. For experimental details see the text.

Fig. 5. (a) Fractionation by gel chromatography on Sepharose 2B of the isolated interacting component and (b) interaction of the subfractions with disaggregated proteoglycan

(a) A sample of the ECTEOLA-cellulose 0.5M-NaCl fraction containing 1mg of uronic acid was applied to a column (165cm×1.1cm) of Sepharose 2B eluted as described in the text. The fractions were analysed for uronic acid and pooled as shown to give three fractions of decreasing size, 1, 2 and 3 respectively. (b) The ability of fractions 1 (●), 2 (■) and 3 (▲) to interact with disaggregated proteoglycan (containing 1mg of uronic acid) was measured by gel chromatography as described in the text.

gradient, a small amount of hyaluronic acid separated from the proteoglycans. Its detailed distribution in the gradient is shown in Fig. 6(a). The gradient was divided into twelve equal fractions and the ability of each to interact with disaggregated proteoglycan was assessed by gel chromatography. There was a fairly broad band of activity in the middle of the gradient. The buoyant density of 90% of the material was between 1.43 and 1.56g/ml. The diffuseness of the band may have resulted from failure to achieve true equilibrium in the gradient, but may also have been contributed to by the polydispersity of the hyaluronic acid. Purified hyaluronic acid from umbilical cord also distributed in the gradient in a similar way (Fig. 6b), although its peak was at a slightly lower buoyant density, i.e. 1.48g/ml compared with 1.51g/ml.

The interaction of hyaluronic acid with proteoglycans, as assessed by gel chromatography, provided a sensitive method for the assay of hyaluronic acid in glycosaminoglycan fractions from cartilage (T. E. Hardingham & H. Muir, unpublished work). Preliminary results showed that pig laryngeal cartilage contained 0.35mg of hyaluronic acid/g wet wt. (accounting for 0.7% of the total uronic acid), and that most of this (about 95%) was associated with purified aggregated proteoglycan. That hyaluronic acid is essential for the formation of aggregates can be deduced from the results of Gregory (1973) as discussed below.

Effect of pH on the extraction of proteoglycan aggregates

Sajdera & Hascall (1969) showed that extraction of cartilage with 4M-guanidinium chloride dissociated proteoglycan aggregates and the proportion that re-formed when the extract was dialysed to low ionic strength depended on the pH of extraction and was maximal at pH5.8. Since hyaluronic acid is essential for the formation of aggregates, the observed effect of pH may have been on the amount of hyaluronic acid extracted. The proportion of aggregates in proteoglycans extracted in 4M-guanidinium chloride between pH3.0 and pH8.5 was therefore assessed and the relative amount of hyaluronic acid in each extract measured. After extraction and before dialysis to 0.5M-guanidinium chloride, the extracts were adjusted to pH5.8, so that in each extract aggregates were re-formed under similar conditions. The proteoglycans were then purified in an associative density gradient and the proportion of aggregates in each preparation was estimated by gel chromatography on Sepharose 2B. The proportion varied with pH as shown in Fig. 7; the proteoglycans extracted at pH4.5 contained the largest proportion of aggregates, amounting to about 75% of the total uronic acid.

The proteoglycans from each extract were then separated into three fractions in a dissociative gradient. The hyaluronic acid content of the middle fractions from the gradient was determined by measuring interaction with a 1.5-fold excess of the bottom fraction of disaggregated proteoglycans extracted at pH5.8. The extent of interaction was similar for all the fractions (Fig. 8), showing that the amount of hyaluronic acid extracted varied little with pH. In contrast, the proteoglycans extracted at different pH values differed widely in their ability to interact with an excess of the hyaluronic acid fraction from the pH5.8 extract (Fig. 8). The proteoglycans extracted at pH4.5 contained the largest proportion able to interact. The proportion of aggregates in extracts prepared at different pH values was thus related to the ability of the

Fig. 6. Equilibrium density-gradient centrifugation under dissociative conditions of (a) purified proteoglycans and (b) hyaluronic acid

Purified proteoglycans from an associative gradient were fractionated in a dissociative CsCl gradient (starting density 1.50 g/ml) containing 4M-guanidinium chloride–0.05M-sodium acetate, pH 5.8, at 95000g_{av} for 48 h at 20°C as described in the text. Twelve fractions (each 1.5 ml) were collected by piercing and draining the tubes. A portion (100 µl) of each fraction was mixed with disaggregated proteoglycan and the interaction (○) measured by gel chromatography as described in the text. The uronic acid (●) and protein (Folin method) contents (■) of each gradient fraction were determined after dialysis against 0.5M-sodium acetate. Density is shown by the dashed line.

proteoglycan to interact with hyaluronic acid and not to the amount of hyaluronic acid in the extract.

The proportion of aggregates obtained was greatest at pH 4.5, which differs from the optimum pH of 5.8 reported by Hascall & Sajdera (1969). The present experiments, however, differ in several details from those of Hascall & Sajdera (1969), the most important being that in the present study the 4M-guanidinium chloride extracts were all adjusted to pH 5.8 before dialysis and hence the ability of all the extracts to form aggregates was tested under identical conditions. In the experiments of Hascall & Sajdera (1969), on the other hand, the proportions of aggregates in each extract were determined at pH 5.8, but only after dialysis at the pH of extraction. Since other experiments showed that aggregates became increasingly dissociated as the pH fell below 5.8 the formation of aggregates may well have been decreased in those extracts dialysed below pH 5.8. That this may be the explanation is shown by a further experiment of Hascall & Sajdera (1969), in which the proportion of aggregates in the extract

prepared at pH 5.8 was much decreased when the pH was adjusted to pH 3.8 before dialysis. The results of Hascall & Sajdera (1969) thus reflect a combination of two effects, (a) the effect of pH on extraction and (b) the effect of pH on reaggregation. The experiments described here were designed to examine only the effect of pH on extraction. The present and previous results were thus comparable at pH 5.8 and above when reaggregation was favoured, but different at lower pH values, when it was inhibited. However, even at low pH the two results could be compatible if the stabilizing influence of the protein-rich fraction (see 'General discussion'), but not the proteoglycan–hyaluronic acid interaction, was adversely affected by low pH, because gel chromatography cannot distinguish between aggregates and the complex resulting from the interaction of proteoglycans with hyaluronic acid.

As with the present results, Hascall & Sajdera (1969) showed that above pH 5.8 there was a progressive decrease in the proportion of aggregates in the extract. Extraction at high pH values appeared

Fig. 7. Gel chromatography on Sepharose 2B of proteoglycans extracted in 4M-guanidinium chloride, pH 3.0–8.5

Proteoglycans extracted in 4M-guanidinium chloride buffered at (a) pH 3.0, (b) pH 4.5, (c) pH 5.8, (d) pH 7.0, (e) 8.5, were purified in an associative gradient at pH 5.8 as described in the text. Samples of the purified proteoglycans containing 1 mg of uronic acid were applied to a column (165cm×1.1cm) of Sepharose 2B eluted as described in the text. The fractions were analysed for uronic acid. V_0 and V_t mark the void volume and total volume of the column respectively.

to affect irreversibly the ability of some proteoglycans to interact with hyaluronic acid. If the factors that bring about this 'denaturation' of proteoglycan can be identified more ideal conditions of extraction may be devised. Under the conditions used in the present study, however, the effects were minimal at pH 4.5 and this pH was therefore used for all subsequent extractions of proteoglycans in 4M-guanidinium chloride.

When hyaluronic acid was present in excess, the proportion of disaggregated proteoglycans that interacted with it never approached the proportion that was excluded from Sepharose 2B in the original aggregated preparation, although the difference was

Fig. 8. Interaction measured by gel chromatography between dissociative density-gradient fractions of proteoglycans extracted at different pH values

Purified proteoglycans extracted at different pH values were fractionated in a dissociative density gradient as shown in Table 1. Samples of the middle fractions were each mixed with a standard preparation of disaggregated proteoglycan (extracted at pH 5.8) present in 1.5-fold excess (relative to the original proportions in the gradient), and the interaction was measured by gel chromatography as described in the text (●). Samples of the bottom fractions were mixed with a standard preparation of middle fraction (from a pH 5.8 extract) present in twofold excess (relative to the original proportions in the gradient) and the interaction was similarly measured by gel chromatography (■). The proportion of aggregates in each preparation before disaggregation (Fig. 8) is shown for comparison (▲).

not always as large as that found in the pH experiments (Fig. 8). This suggested that subsequent treatment of the proteoglycan involving further exposure to 4M-guanidinium chloride and/or CsCl during dissociative gradient centrifugation may have decreased their capacity to interact. Moreover, as the proteoglycans had been extracted in 4M-guanidinium chloride and purified in the presence of concentrated (5M) CsCl before their degree of aggregation was assessed, it could be argued that proteoglycans were more aggregated *in vivo* than in the extracts. On the other hand, there is evidence that not all proteoglycans are aggregated *in vivo*. Proteoglycans of relatively small molecular size have been preferentially extracted from cartilage with iso-osmotic sodium acetate or NaCl (Tsiganos & Muir, 1969a; Brandt & Muir, 1969, 1971; Šimůnek & Muir, 1972). Since these conditions of extraction would not dissociate

aggregates it implies that these smaller proteoglycans were present in cartilage in a non-aggregated form and that they probably account for some of the non-aggregated proteoglycan in the 4M-guanidinium chloride extract. The extraction of the cartilage with iso-osmotic NaCl followed by 4M-guanidinium chloride appeared to be a simple method for separating aggregated and non-aggregated proteoglycans, thus providing a further assessment of their relative abundance and also enabling their characteristics to be examined in more detail.

Preparation of non-aggregated and aggregated proteoglycans

Preliminary experiments showed that extraction with 0.15M-NaCl at 4°C brought into solution a maximum of 12% of the total uronic acid (Fig. 9a), which was largely all in solution after 3h. Subsequent extraction with 4M-guanidinium chloride, buffered at pH4.5, for 24h at 4°C then brought into solution a further 71% of the total uronic acid (Fig. 9b). The combined yield of the two extracts (83%) was thus comparable with that obtained from a single extraction with 4M-guanidinium chloride, suggesting that the population of proteoglycans extracted by the sequential method was similar to the population extracted by a single-step procedure.

Fig. 9. Time-course of extraction of proteoglycans from pig laryngeal cartilage

(a) Weighed samples of sliced cartilage (5.0g) prepared as described in the text were extracted with 50ml of unbuffered 0.15M-NaCl at 4°C. Samples were withdrawn at timed intervals up to 6h and the uronic acid content was measured. (b) The cartilage residue from samples extracted for 3h with 0.15M-NaCl were suspended in 50ml of 4M-guanidinium chloride buffered at pH4.5. Samples were withdrawn at timed intervals and the uronic acid contents measured.

This form of sequential extraction was used to prepare two proteoglycan fractions as shown in Scheme 1. Proteoglycans extracted first (fraction N) resembled those extracted from the same type of cartilage by using 0.15M-sodium acetate with low-speed homogenization (Tsiganos & Muir, 1969b). The protein content was low and galactosamine/glucosamine molar ratio was high (24:1), indicating a low keratan sulphate content (Table 3). Gel chromatography on Sepharose 2B showed a broad distribution of size (K_{av} 0.51; Fig. 10a) and as only 7% was excluded from the gel, few aggregates were present. The proteoglycans extracted subsequently with 4M-guanidinium chloride (fraction A1) had a high protein and keratan sulphate content (Table 3) and consisted of a large proportion of aggregates, since about 90% was excluded from Sepharose 2B (Fig. 10d). This extract was thus enriched in aggregates compared with the single-step 4M-guanidinium chloride extract, presumably because most of the smaller non-aggregated proteoglycans had already been extracted with 0.15M-NaCl.

Further evidence for the aggregated and non-aggregated nature of the proteoglycan fractions was obtained by equilibrium density-gradient centrifugation under dissociative conditions. Proteoglycans in fraction A1 were disaggregated in the gradient and a protein-rich fraction D3 was separated from the majority of the proteoglycans (D1), which were thereby decreased in protein and keratan sulphate content (Table 3). Gel chromatography of the disaggregated proteoglycans showed a single retarded peak on Sepharose 2B (K_{av} 0.24; Fig. 10e), as found with other preparations of disaggregated proteoglycans from non-sequential extracts (see Fig. 1). In contrast, the molecular size of the proteoglycans extracted with 0.15M-NaCl was unaffected by fractionation in a dissociative density gradient (Fig. 10b). Only a small amount of protein separated at the top of the gradient (Fig. 11), so that the composition of the proteoglycans in the bottom fraction was almost unchanged (ND1; Table 3) and the middle fraction (ND2) contained no detectable hyaluronic acid.

Comparison of the non-aggregated proteoglycans (ND1) with the disaggregated fraction (D1) showed that the latter was larger on gel chromatography (K_{av} 0.24 compared with K_{av} 0.50 on Sepharose 2B, Figs. 10b and 10e) and that it contained more protein and keratan sulphate (Table 3). This result is not compatible with the existence of a protein core common to the two groups of molecules, because, on the basis of a simple 'bottle brush' model of proteoglycan structure (Mathews & Lozaityte, 1958; Partridge *et al.*, 1961), size should increase with the number of chondroitin sulphate chains, whence proteoglycans of small size would have a high protein content. As previously proposed (Tsiganos & Muir, 1969b), the low protein content of the small

Scheme 1. Sequential extraction of non-aggregated and aggregated proteoglycans from pig laryngeal cartilage. Fractions ND2 and ND3 were not analysed in detail. The distribution of fraction N in the gradient is shown in Fig. 11.

Table 3. Composition of fractions of proteoglycans extracted from pig laryngeal cartilage

Fractions are as shown in Scheme 1. Protein content is obtained from summation of amino acid analyses.

Fraction	Uronic acid content (% of total in tissue)	Uronic acid/protein (Folin) wt. ratio	Galactosamine/glucosamine molar ratio	Uronic acid content (% of dry wt.)	Protein content (% of dry wt.)
N	12.2	2.23	24.2	25.2	7.21
ND1	11.6	2.48	25.3	26.0	6.88
A1	70.2	1.51	9.5	23.8	11.05
A2	5.9	0.133	4.3	8.8	—
D1	67.3	2.24	12.5	24.9	8.18
D2	2.2	0.40	1.79	15.2	42.0
D3	0.7	0.063	1.10	5.3	79.0

Fig. 10. Gel chromatography on Sepharose 2B of proteoglycans extracted sequentially (see Scheme 1)

(a) Proteoglycan extracted with 0.15M-NaCl (N); (b) fraction N after dissociative gradient fractionation to give fraction ND1; (c) fraction ND1 mixed with 1% (w/w) hyaluronic acid; (d) proteoglycan extracted with 4M-guanidinium chloride (A1); (e) fraction A1 after dissociative gradient fractionation to give fraction D1; (f) fraction D1 mixed with 1% (w/w) hyaluronic acid. Samples containing about 1mg of uronic acid were applied to a column (165cm $\times$ 1.1cm) of Sepharose 2B eluted as described in the text. The uronic acid (—) and protein (Folin method) (----) contents of the fractions were determined. V_0 and V_t mark the void volume and total volume of the column respectively.

non-aggregated proteoglycans implies that they have a smaller protein core than the disaggregated proteoglycans.

The amino acid composition of the non-aggregated and aggregated proteoglycans provided further evidence that the protein cores were different. Although the analyses show a general similarity (Table 4), the non-aggregated proteoglycans consistently contained more serine and glycine and fewer cysteine, tyrosine and arginine residues than did the

Fig. 11. Equilibrium density-gradient centrifugation under dissociative conditions of the proteoglycan extracted with 0.15M-NaCl (N)

The purified proteoglycan in 4M-guanidinium chloride-0.05M-sodium acetate, pH5.8, containing CsCl at a starting density of 1.50g/ml was centrifuged at 95000g_{av} for 48h at 20°C as described in the text. Twelve samples were collected, and the uronic acid (●) and protein (■) contents determined after dialysis against 0.5M-sodium acetate, pH6.8. Density is shown by the dashed line.

disaggregated proteoglycans. The analyses of the other fractions produced by density-gradient fractionation of fraction A1 are also included in Table 4 for comparison. The protein-rich fraction D3 contained much more aspartate, cysteine and aromatic and basic amino acids than the proteoglycan D1, and fraction D2 had an analysis intermediate between that of fractions D1 and D3. Further examination showed that it was not a mixture of the components of D1 and D3, as the analysis was unaltered after re-running in the gradient (T. E. Hardingham & H. Muir, unpublished work). Its analysis and position in the gradient suggests that this fraction contained some proteoglycans of high protein content in addition to hyaluronic acid. Similar differences in amino acid analyses were previously noted between density-gradient fractions of disaggregated proteoglycans extracted in one step (Tsiganos *et al.*, 1971). The present results showed that differences in amino acid composition were still present within the proteoglycan population even when most of the non-aggregating fraction had been removed. The significance of these differences has not yet been examined in detail.

Chondroitin sulphate chains are attached to the protein core of proteoglycans via alkali-labile linkages to serine residues (Muir, 1958). The chains attached to both small and large proteoglycans from pig laryngeal cartilage had been shown to be of similar size (mol.wt. about 15000) (Tsiganos & Muir, 1969b), enabling the proportion of serine residues with chains attached to be calculated from the analyses of the non-aggregated and disaggregated

Table 4. *Amino acid composition of proteoglycan fractions from pig laryngeal cartilage*

Fractions are as shown in Scheme 1. Each value given is the average of two separate analyses. No corrections were applied for losses during hydrolysis.

Fraction	Composition (residues/1000 residues)					
	...	ND1	A1	D1	D2	D3
Asp		68.3	82.9	76.3	93.3	125.8
Thr		56.2	59.7	62.3	62.6	45.7
Ser		150.0	103.8	122.7	73.3	56.5
Glu		145.8	127.5	140.0	121.2	95.1
Pro		79.8	92.2	98.5	83.2	68.5
Gly		142.9	122.0	129.5	94.5	102.1
Ala		71.9	80.1	79.4	81.8	76.2
Cys		Trace	7.5	2.4	13.3	24.4
Val		61.8	58.7	58.9	56.0	53.4
Met		3.9	4.4	3.4	8.3	4.9
Ile		38.8	36.3	36.5	34.3	31.0
Leu		80.7	82.1	76.7	90.7	88.3
Tyr		13.0	27.2	19.8	35.4	42.6
Phe		25.3	29.9	28.0	39.6	42.6
Lys		10.2	21.5	11.5	33.8	50.0
His		8.0	13.7	9.8	19.1	23.9
Arg		29.1	50.1	44.1	59.7	64.5

fractions. Although non-aggregated proteoglycans contained less protein they contained a higher proportion of serine residues, such that the proportion linked to chondroitin sulphate chains was very similar in both, i.e. 52% in the non-aggregated and 49% in the disaggregated proteoglycans. These are, however, average values and some range of variation is likely in each fraction. A similar calculation applied to fraction D2, which contained proteoglycans of high protein content, showed that only 15% of serine residues appeared to have chondroitin sulphate chains attached.

Interaction of proteoglycan fractions with hyaluronic acid

The behaviour on gel chromatography on Sepharose 2B of the non-aggregated and disaggregated proteoglycans when mixed with excess of hyaluronic acid is shown in Figs. 10(c) and 10(f). Disaggregated proteoglycans interacted with hyaluronic acid so that 74% of the uronic acid became excluded from the gel, whereas less than 5% of the non-aggregated proteoglycan interacted either before or after dissociative density-gradient centrifugation, nor did proteoglycans extracted with 0.15M-NaCl interact with hyaluronic acid before cetylpyridinium chloride precipitation. Hence neither exposure to 4M-guanidinium chloride nor cetylpyridinium chloride precipitation was responsible for failure of these proteoglycans to interact.

The fact that proteoglycans extracted with 0.15M-NaCl were not aggregated and unable to interact with hyaluronic acid would explain why they are present in cartilage in a non-aggregated form and, together with their small size, why they were extracted with iso-osmotic NaCl. Presumably they are retained within the cartilage *in vivo* only because of the presence of a membrane, the perichondrium, which limits diffusion out of the tissue.

General discussion

It was previously shown that hyaluronic acid interacted specifically with disaggregated cartilage proteoglycans (Hardingham & Muir, 1972b). The characteristics of this interaction suggested that a large number of proteoglycans were bound to each hyaluronic acid chain, and that each proteoglycan contained only a single binding site. The inhibition of proteoglycan-hyaluronic acid interaction by oligosaccharides derived from hyaluronic acid showed the binding site to have a high affinity for a unit as small as a decasaccharide (Hardingham & Muir, 1973b). The properties of the proteoglycan-hyaluronic acid system were remarkably similar to those of the reversible aggregation of proteoglycan reported by Hascall & Sajdera (1969), the major difference being that the proteoglycan-hyaluronic acid complex did not have a high coefficient of sedimentation in the ultracentrifuge.

Gregory (1973), extending the work of Hascall & Sajdera (1969), presented evidence that two different components were required for the formation of proteoglycan aggregates; one was a fraction rich in protein of low buoyant density (about 1.25g/ml) equivalent to fraction D3 in the present work; the other was a fraction with a buoyant density similar to that of hyaluronic acid. These results, together with the present identification of hyaluronic acid in proteoglycan aggregates, show that the interaction between proteoglycan and hyaluronic acid is essential for the formation of aggregates, which in addition requires the protein-rich fraction D3 to stabilize the complex in some way that alters its behaviour in the ultracentrifuge. Confirmation of the role of hyaluronic acid and the protein-rich fraction in aggregation has also been established in proteoglycans from bovine nasal septa and bovine tracheal cartilage (V. C. Hascall & D. Heinegård, personal communication).

The presence of hyaluronic acid in the middle density-gradient fraction D2 explains why when returned to normal associative conditions for gel chromatography, the proteoglycans in this fraction were largely excluded from Sepharose 2B (Tsiganos *et al.*, 1971), whereas the disaggregated proteoglycans at the bottom of the gradient were not. A similar result was reported in the fractionation of proteoglycans from bovine trachea (Heinegård, 1972a). In the light of this explanation it appears unnecessary to consider that these proteoglycans are basically different from those in the bottom of the gradient.

The glucosamine content of purified proteoglycans has been shown to be attributable mainly to keratan sulphate attached to the protein core (Tsiganos & Muir, 1967; Heinegård & Gardell, 1967), but the present results show that some of the glucosamine in purified aggregates is present in hyaluronic acid. However, it accounted for only a small proportion of the total glucosamine (about 7.0%) and if present in similar amounts in other cartilage would not invalidate the use of glucosamine as a general measure of keratan sulphate content.

Various factors affect the proportion of aggregates in proteoglycan preparations. Thus there is some evidence that the proportion decreased with age and may vary from one type of cartilage to another (Tsiganos & Muir, 1973). Moreover, conditions of isolation are critical, since in addition to the effects of pH shown here, proteoglycan aggregates were unstable to high-speed homogenization or sonication (Sajdera & Hascall, 1969). It would appear that the region of the molecule necessary for aggregation is sensitive to denaturation and is maintained by disulphide bridges, since procedures for the reduction and alkylation of cystine residues abolished the ability to interact with hyaluronic acid (T. E. Hardingham & H. Muir, unpublished work)

and caused disaggregation (Hascall & Sajdera, 1969).

Small non-aggregated proteoglycans are not peculiar to one type of cartilage. They have previously been characterized from pig laryngeal cartilage (Tsiganos & Muir, 1969b), pig articular cartilage (Brandt & Muir, 1971; Šimůnek & Muir, 1972) and bovine nasal septum (Mayes *et al.*, 1973). Brandt *et al.* (1973) showed that small proteoglycans, extracted with 0.15M-sodium acetate, reacted directly with antiserum raised against whole proteoglycans, whereas larger proteoglycans (including disaggregated fractions) required hyaluronidase digestion before any interaction was possible. Thus although small and large proteoglycans shared most antigenic determinants, these were more accessible in the former.

The inability of non-aggregated proteoglycans to interact with hyaluronic acid suggests that they are structurally different from those able to aggregate. Moreover, comparison of their analysis and hydrodynamic size is incompatible with the presence of a protein core common to both types of proteoglycan. Further, they do not appear to have resulted from degradation of larger proteoglycans either *in vivo* or during preparation. There was no detectable proteolytic activity in iso-osmotic sodium acetate extracts of cartilage (Tsiganos & Muir, 1969a) and there was a limit to the amount of non-aggregated proteoglycans extracted with time (Fig. 9), which suggested that they were not being formed by enzyme activity during the extraction procedure. Hardingham & Muir (1972a), using pulse-chase experiments, showed that smaller proteoglycans were neither precursors nor degradation products of large proteoglycans, both being synthesized at the same time and independently of each other, although possible relationships within the chondrocytes during their formation have not yet been ruled out.

All preparations of cartilage proteoglycans are very polydisperse. This is partly the result of extensive variation in the number and type of polysaccharide chains attached to the protein core (see Muir, 1969). Whether it also arises from the existence of different protein cores remains a source of controversy. There is evidence both for and against this proposal and it is apparent that the type of preparation studied and the methods used to examine it are most important (as discussed by Tsiganos & Muir, 1969b; Hascall & Sajdera, 1970). Since fractionation in a 'dissociative' gradient separates non-covalently bound protein from purified proteoglycans little reliance can be put on deductions made with the use of material in which the possibility of similarly bound protein has not been excluded.

It was suggested that proteoglycans extracted from bovine nasal septum in 4M-guanidinium chloride and subfractionated in a dissociative density gradient

consisted of a single polydisperse species (Hascall & Sajdera, 1970). A similar conclusion was drawn when chondroitinase-digested proteoglycans were fractionated in a similar way (Hascall & Riola, 1972). On the other hand, 0.15M-KCl extracted 15–20% of the total proteoglycans from the same type of cartilage; these proteoglycans had a different protein content and amino acid analysis from the majority extracted with 4M-guanidinium chloride (Mayes *et al.*, 1973). This suggested that dissociative gradient centrifugation used by Hascall & Sajdera (1970), which fractionates molecules largely according to their relative carbohydrate/protein content, had failed to detect these differences. This may be because each proteoglycan species varies in its relative carbohydrate/protein content and thus overlaps extensively in the gradient. Other techniques such as zone electrophoresis (Mashburn & Hoffman, 1971; Mayes *et al.*, 1973) also failed to detect some aspects of heterogeneity revealed by other methods such as sequential extraction or gel chromatography, thus emphasizing the importance of the methods chosen to examine this problem.

The existence of proteoglycans of aggregating and non-aggregating types in many varieties of cartilage suggests that there are a minimum of two types of protein core. Further, examination of the protein cores of proteoglycans from bovine tracheal cartilage digested with hyaluronidase and fractionated by gel chromatography revealed at least three fractions (Heinegård, 1972b). The differences in amino acid composition among disaggregated proteoglycan fractions reported here (i.e. D1 and D2) also suggests that there were more than two types of core protein.

Although there is considerable homology in protein structure between proteoglycans prepared by similar methods from cartilage from different anatomical sites and species (Mathews, 1971), this is not incompatible with the existence of more than one type of proteoglycan, because homology could apply equally well to a group of polypeptides as to a single species. The existence of distinct types of proteoglycan suggests that age-related and pathological changes in proteoglycan composition may have two separate origins; one may be the result of preferential synthesis of particular proteoglycan cores, the amino acid sequence of which may determine the number and type of polysaccharide chains attached (see Baker *et al.*, 1972); the other may involve some change in the production and organization of the enzymes and substrates necessary for polysaccharide chain synthesis (see Rodén, 1970). Such changes in composition could lead to changes in the quality of the proteoglycans, such as alterations in the proportion and size of the aggregates they form, the importance of which needs to be evaluated in aged and diseased tissue.

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