Proteoglycan Populations of Baboon (*Papio papio*) Articular Cartilage

GEL-ELECTROPHORETIC ANALYSIS OF FRACTIONS OBTAINED BY DENSITY-GRADIENT CENTRIFUGATION AND BY SEQUENTIAL EXTRACTION

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1. Gel electrophoresis of proteoglycans extracted with use of 4M-guanidinium chloride from baboon (*Papio papio*) articular cartilage and purified on DEAE-cellulose in 8M-urea yielded three bands on electrophoresis in polyacrylamide/agarose gels: two wide bands close together (I and II) and a third, thinner and more rapidly moving band (III). 2. Gel electrophoresis of fractions from direct 'dissociative' gradients showed that these bands were partially separated (buoyant density of I > II > III). 3. Reduction and alkylation of proteoglycans did not alter either the gel-electrophoretic pattern or the distributions of the bands in the fractions of the gradient. 4. Band III was found in the upper third of 'associative' gradients but not in the bottom fraction, which yielded after dissociation only bands I and II. 5. The third band was completely extracted for 24h with an iso-osmotic solution, but was contaminated with bands I and II. The second extraction step with 4M-guanidinium chloride yielded only bands I and II. 6. The data strongly suggest the presence in the articular cartilage of several populations of dissociated proteoglycans differing in gel-electrophoretic migration, buoyant density and aggregation capacity.

Proteoglycans of cartilage consist of chains of chondroitin sulphate and keratan sulphate covalently linked to protein(s), part of which interacts specifically with hyaluronic acid (Hardingham & Muir, 1974) with the formation of aggregates. The aggregates are stabilized by two 'link' proteins (Hardingham & Muir, 1974), but may be dissociated in the presence of 4M-guanidinium chloride into proteoglycan 'sub-units'. Dissociated proteoglycans are polydisperse and heterogeneous and exhibit variations in aggregation capacity. In previous studies we found that dissociated proteoglycans of articular cartilage from pig, baboon and human, which had been extracted with guanidinium chloride and purified on DEAE-cellulose, were heterogeneous as revealed by gel electrophoresis (Stanescu *et al.*, 1973; Stanescu & Maroteaux, 1975a).

In the present study a gel-electrophoretic analysis was applied to fractions of cartilage proteoglycans that were separated by equilibrium-density-gradient centrifugation under 'associative' and 'dissociative' conditions. Gel-electrophoretic analysis was also applied to proteoglycan fractions obtained by sequential extraction with 0.15M-NaCl, followed by extraction with 4M-guanidinium chloride. The results showed that the dissociated proteoglycans of baboon articular cartilage appear to be of three main groups or types, differing in gel-electrophoretic migration, extractability and buoyant density.

Materials and Methods

Chemicals

Urea ('for biochemistry' grade) was from Merck, Darmstadt, West Germany. Guanidinium chloride (ultra pure) was from Schwarz/Mann, Orangeburg, NY, U.S.A.; DEAE-cellulose (microgranular form) was from Whatman, Ferrières, France; benzamidinium chloride and 6-aminohexanoic acid ('for synthesis' grade) were from Merck; soya-bean trypsin inhibitor was from Worthington, Freehold, NJ, U.S.A.; EDTA (disodium salt) ('for analysis' grade) and CsCl were from Merck; pepstatin was from Banyu Pharmaceutical Co., Tokyo, Japan; acrylamide and bisacrylamide were from Canalco, Rockville, MD, U.S.A.; agarose (Indubiose A 37) was from Industrie Biologique Française, Gennevilliers, France. Unless otherwise stated, all other chemicals used were of analytical grade.

Methods

Articular cartilage was isolated from young adult baboons (*Papio papio*) which were obtained from the Institut Pasteur, Garches, France. The large joints of the limbs, obtained within a few minutes of death of the animals, were frozen in solid CO₂ and stored at -60°C. After thawing to 4°C the joints were opened and the fluid was wiped away. The articular cartilage

was shaved, diced very finely with a scalpel and briefly rinsed with 0.9% NaCl at 4°C.

Extraction of proteoglycans. The cartilage pieces were directly transferred to and extracted in ten times their weight of cold 4M-guanidinium chloride (Sajdera & Hascall, 1969) by gentle shaking for 48 h in a Fisher Roto-Rack. The guanidinium chloride was buffered to pH 7.4 with 0.05M-Tris/HCl, to pH 5.8 with 0.05M-sodium acetate and to pH 4.5 with sodium citrate/sodium phosphate buffer (McIlvaine, 1921) diluted 1:4 (v/v) (Hardingham & Muir, 1974). In an attempt to inhibit the proteolytic activity present in the extract the following substances were added: 0.01M-EDTA (disodium salt), 0.1M-6-amino-hexanoic acid, 5mM-benzamidinium chloride, 1mg of soya-bean trypsin inhibitor/litre, 10µg of pepstatin/litre. The pH was adjusted after the addition of inhibitors. The extract was then centrifuged at 36900g (r_{av} , 3.25cm) for 20min and the pellet was washed several times with cold 4M-guanidinium chloride containing proteolysis inhibitors. The 4M-guanidinium chloride extract contained 72–75% of the total tissue hexuronate.

Purification of proteoglycans by ion-exchange chromatography on DEAE-cellulose (Antonopoulos *et al.*, 1974). After dialysis against 8M-urea in 0.05M-Tris/HCl buffer, pH 6.8 at 4°C, the solutions were chromatographed on columns of DEAE-cellulose (Cl⁻ form) that had been equilibrated with 8M-urea in 0.05M-Tris/HCl buffer, pH 6.8. The dimensions of the columns were chosen according to the amount of hexuronic acid present in the fractions. Columns were eluted with 3 bed-volumes each of 8M-urea, 0.3M-NaCl in 8M-urea and 2M-NaCl in 8M-urea. The last fraction contained most of the proteoglycans (about 80–85% of the hexuronic acid) and this was used for further analysis. All the urea solutions contained proteolysis inhibitors as described above.

Equilibrium-density-gradient-centrifugation procedures. (a) One-step fractionation of proteoglycans under 'dissociative' conditions in 4M-guanidinium chloride. In several experiments the purification of extracted proteoglycans by chromatography on DEAE-cellulose was performed as described above, then the 2M-NaCl fraction was dialysed against 4M-guanidinium chloride and prepared for the gradient. In other experiments the 4M-guanidinium chloride extract was fractionated on the gradient and the fractions containing proteoglycans were purified on DEAE-cellulose columns as described above. Gradients were prepared by adding solid CsCl to a density of 1.50g/ml. Centrifugation was at 130300g (r_{av} , 5.7cm) for 48h at 18°C in a Spinco type-65 rotor in 8×13ml polyallomer tubes. After centrifugation, the tubes were quickly frozen in liquid N₂ and cut into five or six sections (Hardingham & Muir, 1974).

An Auto Densiflow II C Buchler instrument was used for the isolation of a larger number of fractions. Any insoluble gel present in the top fraction was removed with a spatula. Densities of the fractions were determined by weighing samples in a 100µl constriction pipette. The uronic acid content was determined as described by Bitter & Muir (1962) in 0.1–0.25ml samples (diluted if necessary), and the protein content was determined by a micro-method based on the procedure of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

(b) Fractionation of proteoglycans under 'associative' conditions followed by fractionation under 'dissociative' conditions. The extracts of cartilage in 4M-guanidinium chloride were dialysed against 9vol. of the corresponding buffer for 12h at 4°C, then adjusted to a density of 1.65g/ml by the addition of solid CsCl. Equilibrium-density-gradient centrifugation was performed at 130300g (r_{av} , 5.7cm) for 48h at 18°C in a Spinco type-65 rotor. The quickly frozen tubes were cut into three equal sections. The bottom third was conserved for analysis in a subsequent 'dissociative' gradient, and the upper and middle sections of the gradients were analysed for hexuronic acid (Bitter & Muir, 1962), purified on DEAE-cellulose as above and examined by gel electrophoresis (see the next paragraph). The experiment was repeated three times. Another gradient was separated into eight fractions by using the Buchler Auto Densiflow apparatus. The bottom quarter was conserved for subsequent dissociation and the other fractions were analysed as described above. An equal volume of 7.6M-guanidinium chloride in the corresponding buffer was added to the bottom fraction, the density was adjusted to 1.50g/ml by the addition of solid CsCl, and the proteoglycan concentration was decreased by adding 4M-guanidinium chloride/CsCl solution ($\rho = 1.50$ g/ml). The solution was centrifuged as described above, and the tubes were quickly frozen and cut into five fractions. In another experiment seven fractions were obtained by using a Densiflow apparatus. The density, the hexuronic acid content and protein content were determined as described above.

Gel-electrophoretic procedure. All samples were dialysed against 8M-urea in 0.05M-Tris/HCl buffer, pH 6.8, before gel-electrophoretic analysis on large-pore composite polyacrylamide/agarose gels. The method of McDevitt & Muir (1971), slightly modified (1.2%, w/v, acrylamide and 0.7% agarose), was used. Samples of proteoglycans equivalent to 1.2–1.5µg of hexuronic acid were layered on each gel. The electrophoresis was performed in the cold-room for 55min (4mA/tube, voltage gradient about 21 V/cm). The gels were stained with 0.2% Toluidine Blue in 0.1M-acetic acid and with Coomassie Blue. Each sample was analysed in two gels and in several runs. A sample of chondroitin sulphate was used as a

standard. Its mobility was greater than that of proteoglycans and was identical on different gels.

Reduction of proteoglycans. In one experiment the proteoglycans obtained in the 2M-NaCl fraction of DEAE-cellulose ion-exchange chromatography were reduced and alkylated before fractionation by ultracentrifugation and gel-electrophoresis analysis. The reduction and alkylation were performed as described by Hascall & Sajdera (1969).

Sequential extraction of proteoglycans with 0.15M-NaCl followed by 4M-guanidinium chloride. Sliced cartilage was frozen, pulverized and then extracted in ten times its weight of 0.15M-NaCl (containing proteolysis inhibitors) for 24h at 4°C by gentle shaking. The extract was filtered on glass-wool. The 0.15M-NaCl extract contained 10–12% of the total tissue hexuronate. After dialysis against 8M-urea in 0.05M-Tris/HCl buffer, pH 6.8, at 4°C, the proteoglycans were purified by chromatography on DEAE-cellulose and submitted to gel electrophoresis as described above. The washed cartilage residue from the extraction with 0.15M-NaCl was suspended in ten times its weight of 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, and extracted for 48h at 4°C. The extracts were dialysed against 8M-urea, pH 6.8, purified on DEAE-cellulose and submitted to gel electrophoresis as above.

Results

Previously dissociated articular-cartilage proteoglycans purified by DEAE-cellulose chromatography were heterogeneous when examined by gel electrophoresis (Stanescu *et al.*, 1973; Stanescu & Maroteaux, 1975a). Equilibrium-density-gradient centrifugation (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969) has been widely used for purification and fractionation of cartilage proteoglycans, and sequential extraction (Tsiganos & Muir, 1969; Hardingham & Muir, 1974) has also been used to obtain different fractions of proteoglycan. In the present study, proteoglycans from baboon articular cartilage were fractionated by equilibrium-density-gradient centrifugation and by sequential extraction, purified by DEAE-cellulose chromatography and examined by gel electrophoresis. The preparative sequence of the different fractions is represented in Scheme 1.

Gel electrophoresis of non-fractionated proteoglycans extracted with 4M-guanidinium chloride at pH 7.4 or 5.8 and purified on DEAE-cellulose

The Toluidine Blue staining showed three bands: two wide strongly metachromatic bands close

A. Purified disaggregated proteoglycans

Extraction with 4M-guanidinium chloride and with proteolysis inhibitors (48h at 4°C) → DEAE-cellulose in 8M-urea → Gel electrophoresis

B. Fractions separated by sequential extractions

Extraction with 0.15M-NaCl and with proteolysis inhibitors (24h at 4°C) → DEAE-cellulose in 8M-urea → Gel electrophoresis

Second extraction with 4M-guanidinium chloride and with proteolysis inhibitors (48h at 4°C) → DEAE-cellulose in 8M-urea → Gel electrophoresis

C. Fractions separated by density-gradient centrifugation

Extraction with 4M-guanidinium chloride and with proteolysis inhibitors (48h at 4°C) → DEAE-cellulose in 8M-urea → CsCl density gradient in 4M-guanidinium chloride → DEAE-cellulose in 8M-urea of the fractions → Gel electrophoresis

↓

CsCl density gradient in 0.4M-guanidinium chloride → DEAE-cellulose in 8M-urea of fractions A₃–A₈ → Gel electrophoresis

↓

Second CsCl density gradient in 4M-guanidinium chloride of the bottom third of associative gradient → DEAE-cellulose in 8M-urea of the fractions → Gel electrophoresis

Scheme 1. Preparative sequence of the different fractions analysed by gel electrophoresis

together (I and II) and one thin, more rapidly moving, metachromatic band (III) (Plate 1a). The two main bands were very faintly stained by Coomassie Blue and band III was well stained. The gel-electrophoresis results were similar for proteoglycans extracted at pH 7.4 or at 5.8. No material was found at the origin of the gels.

Gel electrophoresis of proteoglycans extracted with 4M-guanidinium chloride (pH 7.4 or 5.8), purified on DEAE-cellulose and fractionated by equilibrium-density-gradient centrifugation under 'dissociative' conditions

The 'dissociative' gradient was cut into five fractions (for details, see under 'Methods'), and the densities and the distribution of uronic acid and protein are shown in Table 1.

The gel-electrophoretic analysis showed three bands with the same migration and appearance as those found for the unfractionated proteoglycans. However, the distribution of each band was different for the various fractions of the gradient (Table 1, Expt. A, and Plate 2b). In the first fraction ($\rho > 1.60$ g/ml) band I was intensely stained, band II was less densely stained, and band III was absent. In the second fraction ($\rho = 1.53$ g/ml) all three bands were present, but band II was most densely stained. In fraction 3 ($\rho = 1.48$ g/ml) and fraction 4 ($\rho = 1.44$ g/ml) band III was predominant, band I was absent and band II was very faint. In fraction 5 ($\rho < 1.39$ g/ml) only band III was found.

In another experiment the distribution of material through the gradient was examined in more detail. An initial density of 1.52 g/ml was used and six

fractions were obtained. Band I was predominant in the fractions with $\rho > 1.73$ g/ml, band II in the fractions with $\rho = 1.68$ and 1.61 g/ml and band III in the fractions with $\rho < 1.54$ g/ml, with some overlap of the bands (Table 1, Expt. B, and Plate 2a).

Reversing the sequence of fractionation of the proteoglycans by carrying out equilibrium-density-gradient centrifugation before DEAE-cellulose chromatography did not alter the results.

Reduction and alkylation of the proteoglycans after DEAE-cellulose chromatography and before equilibrium-density-gradient centrifugation also had no effect on the pattern of gel electrophoresis or the distribution of the bands in the fractions of the gradient.

Gel electrophoresis of proteoglycans extracted with 4M-guanidinium chloride (pH 5.8 or 4.5) fractionated on equilibrium-density-gradient centrifugation under 'associative' conditions and purified on DEAE-cellulose

The upper and middle portions of the 'associative' gradient (Plate 3a) were analysed, and the lower portion containing the aggregates was kept for a second analysis under 'dissociative' conditions. The fractions were dialysed against 8M-urea, pH 6.8, and purified on DEAE-cellulose, then examined by gel electrophoresis. Band III was present in the upper third of the gradient and bands I and II were present in traces (Plate 1b). In the middle third very little material was present, but all three bands were found. The results were similar for four associative gradients in which the extraction pH was 5.8 or 4.5.

Table 1. *Direct dissociative equilibrium-density gradient of proteoglycans extracted with 4M-guanidinium chloride and purified on DEAE-cellulose*

In Expt. A, fractions were obtained by cutting the frozen tubes. The results of the gel-electrophoretic analysis of the fractions are shown in Plate 2(b). In Expt. B, fractions were obtained by using an Auto Densiflow apparatus. The results of the gel-electrophoretic analysis of the fractions are shown in Plate 2(c).

Expt. A

Fraction	Vol. (ml)	Density (ρ) (g/ml)	Uronic acid (% of total)	Protein (% of total)	Protein/uronic acid ratio
1	1.3	1.60	88.33	33.50	0.16
2	1.3	1.53	5.12	17.50	1.56
3	1.3	1.48	2.66	12.50	2.08
4	1.3	1.44	1.74	18	4.60
5	1.5	1.39	2.15	18.50	3.80

Expt. B

Fraction	Vol. (ml)	Density (ρ) (g/ml)	Uronic acid (% of total)
1	1.5	1.73	82.1
2	1	1.68	3.4
3	2	1.61	3.7
4	2	1.54	3.3
5	2	1.49	3
6	2.5	1.43	4.8

EXPLANATION OF PLATE I

Electrophoretic separations of fractions obtained by (a) sequential extractions of proteoglycans and (b) equilibrium density-gradient ultracentrifugation under 'associative' conditions

(a) Proteoglycans were extracted first with 0.15M-NaCl for 24h and then with 4M-guanidinium chloride for 48h. Each fraction was purified on DEAE-cellulose in 8M-urea (Antonopoulos *et al.*, 1974). Electrophoresis was carried out by the method of McDevitt & Muir (1971). (A) Electrophoretic pattern of proteoglycans extracted by 4M-guanidinium chloride; (B) electrophoretic patterns of proteoglycans extracted by sequential extractions with 0.15M-NaCl (1) or with 4M-guanidinium chloride (2). (b) Proteoglycans were extracted with 4M-guanidinium chloride and submitted to an 'associative' equilibrium density gradient of 130300g (r_{av} . 5.7cm) for 48h at 18°C in 0.4M-guanidinium chloride. Three equal fractions were obtained by freezing the centrifuge tube and then cutting it. The proteoglycans were purified on DEAE-cellulose in 8M-urea (Antonopoulos *et al.*, 1974). Electrophoresis was carried out by the method of McDevitt & Muir (1971). (A) Electrophoretic pattern of unfractionated proteoglycans; (B) electrophoretic pattern of the top fraction.

EXPLANATION OF PLATE 2

Electrophoretic separations of fractions obtained by equilibrium-density-gradient ultracentrifugation under direct 'dissociative' conditions

The proteoglycans were extracted with 4M-guanidinium chloride and purified on DEAE-cellulose in 8M-urea (Antonopoulos *et al.*, 1974). The centrifugation was performed at 130300g for 48h at 18°C in 4M-guanidinium chloride. Electrophoresis was carried out by the method of McDevitt & Muir (1971). (a) Electrophoretic pattern of unfractionated proteoglycans; (b) electrophoretic separations of fractions 1-5 of a gradient (see Table 1, Expt. A); (c) electrophoretic separations of fractions 1-6 of a gradient obtained by a different fractionation (see Table 1, Expt. B).

EXPLANATION OF PLATE 3

Electrophoretic separations of fractions obtained by equilibrium-density-gradient ultracentrifugation under 'associative' conditions and under 'associative-dissociative' conditions

The proteoglycans were extracted with 4M-guanidinium chloride and submitted to an associative equilibrium density gradient of 130300g for 48 h at 18°C in 0.4M-guanidinium chloride. Eight equal fractions were obtained by using an Auto Densiflow apparatus. (a) Six upper fractions were purified on DEAE-cellulose (Antonopoulos *et al.*, 1974) and analysed by gel electrophoresis (McDevitt & Muir, 1971). Electrophoretic patterns of the fractions denoted 3-8 in Table 2 are shown. (b) Two lower fractions were submitted to a second 'dissociative' gradient fractionated in five fractions (see Table 3). The proteoglycans of each fraction were purified on DEAE-cellulose in 8M-urea (Antonopoulos *et al.*, 1974) and analysed by gel electrophoresis (McDevitt & Muir, 1971). Electrophoretic patterns of the three lower fractions denoted 1, 2 and 3 are shown.

Table 2. *Associative equilibrium density gradient of proteoglycans extracted with 4M-guanidinium chloride*

Fractions were obtained by using an Auto Densiflow apparatus. The results of the gel-electrophoretic analysis of the fractions are shown in Plate 3(a).

Fraction	Vol. (ml)	Density (ρ) (g/ml)	Uronic acid (% of total)
1	1.4	1.80	81.50
2	1.4	1.74	8.10
3	1.4	1.70	2.40
4	1.4	1.66	1.70
5	1.4	1.64	1.30
6	1.4	1.60	1.40
7	1.4	1.56	1.60
8	1.4	1.53	1.80

Another associative gradient was divided into eight fractions. The two lower fractions were used for subsequent dissociative fractionation. The upper six fractions (Table 2) were analysed and all three bands were found: band I was very faint, band II predominated in the higher-density fractions and band III in the lower-density fractions (Plate 3a). The upper limit of the buoyant-density range of band III seemed to be higher than that found under 'dissociative' conditions.

Gel electrophoresis of proteoglycans extracted with 4M-guanidinium chloride (pH 5.8 or 4.5), purified by equilibrium-density-gradient centrifugation under 'associative' conditions and fractionated in a second 'dissociative' gradient

The fractions from the second dissociative gradient were purified on DEAE-cellulose and analysed by gel electrophoresis.

One of these second gradients is presented in Table 3. Bands I and II were present, but band III was absent from fractions 1-3 with densities greater than 1.73, 1.64 and 1.57 g/ml (Plate 3b). Not enough material was present in the upper fractions to allow analysis. Another faint and slightly metachromatic rapidly moving band was observed in the material obtained from dissociated aggregates. Its position and density distribution were quite different from those of band III.

Gel electrophoresis of proteoglycans obtained by sequential extractions with 0.15M-NaCl followed by 4M-guanidinium chloride and purified on DEAE-cellulose

The proteoglycans extracted with 0.15M-NaCl for 24h at 4°C gave the three bands (I, II, III) on gel electrophoresis; band III was very densely

Table 3. *Associative-dissociative equilibrium density gradient of proteoglycans extracted with 4M-guanidinium chloride*

Fractions were obtained by using an Auto-Densiflow apparatus. The results of the gel-electrophoretic analysis of the fractions are shown in Plate 3(b).

Fraction	Vol. (ml)	Density (ρ) (g/ml)	Uronic acid (% of total)
1	0.6	1.73	81
2	1.6	1.64	12.8
3	1.6	1.57	2.2
4	2.5	1.49	2.5
5	2.7	1.40	1.3

stained. The proteoglycans subsequently extracted with 4M-guanidinium chloride contained only bands I and II.

Discussion

The detection of heterogeneity of dissociated cartilage proteoglycans depends on the methods of fractionation used and the parameters by which heterogeneity is ascertained. The nature of the starting material (anatomical site, species, age) as well as the methods of extraction and purification should also be considered.

It was suggested that dissociated proteoglycans represent a single, albeit polydisperse, population of molecules (Hascall & Sajdera, 1969, 1970). Proteoglycans from bovine nasal cartilage, purified by equilibrium gradient centrifugation under 'associative' conditions, followed by a 'dissociative' gradient, produced proteoglycan 'subunits' with a continuous distribution of sedimentation coefficients. However, the fractions of the second gradient differ in their percentage of chondroitin sulphate, keratan sulphate and protein and amino acid composition (Tsiganos *et al.*, 1971; Heinegård, 1972a; Hardingham & Muir, 1974; Rosenberg *et al.*, 1975a). Studies by Hascall & Heinegård (1974a,b), Heinegård & Hascall (1974) and Rosenberg *et al.* (1975a) suggested that the protein core of proteoglycans contains a constant region (including hyaluronic acid-binding sites) and a variable region with repeating peptide sequences to which chondroitin sulphate chains are attached. This hypothesis found some support in the electron-microscopic studies of the molecules (Rosenberg *et al.*, 1975b; Thyberg *et al.*, 1975) obtained by double gradient purification. Other techniques, such as zone electrophoresis (Mashburn & Hoffman, 1971; Mayes *et al.*, 1973) and rate zonal sedimentation (Hoffman *et al.*, 1975), failed to detect distinct populations of dissociated proteoglycans.

However, other methods allowed separation of different populations of dissociated proteoglycans. A small proportion of proteoglycans in cartilage, extracted with solutions of low ionic strength, are of smaller hydrodynamic size, contain less keratan sulphate and protein and have a different amino acid composition from most of the proteoglycans obtained during a subsequent high-ionic-strength extraction (Tsiganos & Muir, 1969; Brandt & Muir, 1971; Šimůnek & Muir, 1972a; Mayes *et al.*, 1973; Hardingham & Muir, 1974; Lohmander, 1975). This does not seem to be compatible with the proposed model of Heinegård & Hascall (1974) and of Rosenberg *et al.* (1975a), where carbohydrate content should decrease with size (Baxter & Muir, 1975). Proteoglycans extracted with low-ionic-strength solutions are predominantly non-aggregated, are not associated with hyaluronic acid, are not linked to protein and are unable to interact with hyaluronic acid (Hardingham & Muir, 1974). The immunological properties of proteoglycans with the smallest hydrodynamic size differ from those of larger proteoglycans (Brandt *et al.*, 1973). Non-aggregated proteoglycans are probably not precursors or degradative products of larger proteoglycans, since molecules of all sizes are synthesized at the same time (Hardingham & Muir, 1972). Studies of the protein-keratan sulphate core of proteoglycans showed that several fractions which differed in amino acid composition were separated by gel chromatography and gel electrophoresis (Heinegård, 1972b; Baxter & Muir, 1975). However, a single polydisperse core was suggested in another experiment (Hascall & Riolo, 1972; Hascall *et al.*, 1972). Heterogeneity of proteoglycans was found by zone electrophoresis (Muir & Jacobs, 1967; Pedrini, 1969).

Our study strongly suggests that proteoglycans extracted by dissociative procedures from the articular cartilage and purified on DEAE-cellulose contain three different populations. These are clearly separated by gel electrophoresis, and partially separated by equilibrium-density-gradient centrifugation or by sequential extractions. As 'dissociative' gradient centrifugation separates molecules mainly according to their relative carbohydrate/protein content, this result suggests that the three proteoglycan populations also differ in this respect. However, it is probable that each of the three proteoglycan species separated by gel electrophoresis has a variable relative carbohydrate/protein content, and thus they partially overlap in the gradient.

Proteoglycans purified in an 'associative' gradient and then analysed in a second 'dissociative' gradient contained bands I and II but not band III. The latter band was present in the upper half of the first 'associative' gradient. Thus, when proteoglycans were separated by 'associative-dissociative' equilibrium gradients, a special small population of

articular proteoglycans was lost. This was not the case with proteoglycans purified on DEAE-cellulose, since all three bands were detectable in such preparations. It is relevant that Antonopoulos *et al.* (1974) found that the ion-exchange-purified fraction of cartilage proteoglycans contained a greater percentage of the uronic acid in the extract than did the proteoglycan fraction obtained by two equilibrium-density-gradient centrifugations.

The gel-electrophoretic analysis of proteoglycan fractions obtained by sequential extractions in the presence of proteolysis inhibitors demonstrated that band III was completely extracted in 0.15M-NaCl after 24h. However, the extract also contained some band-I and band-II proteoglycans. The fraction obtained by subsequent extraction with 4M-guanidinium chloride contained only band-I and band-II proteoglycans.

These data suggest that band III represents non-aggregated proteoglycans extracted with low-ionic-strength solutions. Small amounts of the material in bands I and II also seem to be non-aggregate, but most of this material appears to form the type of aggregate isolated by 'associative' gradient centrifugation.

After DEAE-cellulose purification of proteoglycans in 8M-urea, no material was found at the origin of the gels, which demonstrated that little or no aggregation occurred, since aggregates are excluded from the gel (McDevitt & Muir, 1971, 1974; V. Stanescu, unpublished work). Similarly, Antonopoulos *et al.* (1974) showed that the gel-chromatographic pattern of the cartilage proteoglycans isolated by the same procedures was identical with that of 'dissociated' proteoglycans obtained by the method of Hascall & Sajdera (1969).

It is unlikely that the heterogeneity of proteoglycans is due to alterations occurring during extraction, purification or fractionation procedures, since these were performed in highly dissociative solutions, at 4°C, and in the presence of proteolysis inhibitors.

The biological significance of the proteoglycan populations should be studied, since age, species and anatomical-site differences have been found. The smaller proteoglycans increase in proportion during early postnatal development of pig articular cartilage (Brandt & Muir, 1971; Šimůnek & Muir, 1972a). Stanescu & Maroteaux (1975a) found that proteoglycans extracted from foetal articular and growth cartilage of several species examined by gel electrophoresis yielded a single band which was slightly slower than the band(s) given by tibial cartilage from older animals. In human growth cartilage the pattern of the foetal proteoglycans changed to the pattern found in older children in the first months of extra-uterine life. Differences were found between the proteoglycans of several species. Proteoglycans from growth cartilage of

humans, baboons, and pigs yielded two wide bands close together (I and II), the pig material migrating more slowly. Proteoglycans of rat and rabbit growth cartilage gave a single band, the rabbit material migrating more slowly. Differences were also found between growth and articular-cartilage proteoglycans of young animals. The gel electrophoresis of the proteoglycans of articular cartilage yielded bands with a migration similar to those given by growing cartilage, but a supplementary fast-moving thin band was found (band III). Its migration was not identical in all the species studied. (Stanescu *et al.*, 1973, 1975). Pathological changes in cartilage proteoglycan populations were observed in the articular cartilage of lame pigs (Šimůnek & Muir, 1972b) and in the growth cartilage of some human chondrodysplasias (Stanescu & Maroteaux, 1975b).

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