The Proteoglycans of the Human Intervertebral Disc

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The methods of Hascall & Sajdera (1969) were used to compare the proteoglycans of human intervertebral disc with those of bovine nasal cartilage. In contrast with cartilage, most of the hexuronate of disc could be extracted at low shear with water or dilute salt solutions. Extracts of disc with 4M-guanidinium chloride were centrifuged in 0.4M-guanidinium chloride in a CsCl gradient. Analytical ultracentrifugation of the hexuronate-containing heavy component revealed two fractions, both more polydisperse than those of cartilage. Also the more rapidly sedimenting component was a much smaller fraction of the total. After prior extraction with 0.4M-guanidinium chloride, 4M-guanidinium chloride extracts of disc were found, by ultracentrifugal analysis, to be enriched in components resembling the proteoglycan monomer and aggregating factors of cartilage.

The proteoglycans isolated from intervertebral discs of cattle and whales comprise mainly chondroitin 6-sulphate and keratan sulphate linked to a common protein core (Rosenberg *et al.*, 1967; Ludowieg *et al.*, 1972). The proteoglycans of human disc appear to be similar, although definitive studies appear not to have been published (Davidson & Woodhall, 1959; Lyons *et al.*, 1964). Preparations from bovine disc, on examination in the analytical ultracentrifuge, showed at least two components with distinct sedimentation behaviour (Rosenberg *et al.*, 1967). In all of these respects, the proteoglycans of disc resemble those of bovine nasal cartilage, the major differences being higher ratios of chondroitin 6-sulphate/chondroitin 4-sulphate and of keratan sulphate/chondroitin sulphates.

High-shear homogenization, although essential for effective extraction with mild reagents, degraded the proteoglycans of cartilage (Harrington & Zimm, 1965; Sajdera & Hascall, 1969). This problem was resolved by the observation that 3M-MgCl₂ and 4M-guanidinium chloride were effective extractants at low shear (Sajdera & Hascall, 1969). The two components observed by ultracentrifugation of such extracts were shown to be a proteoglycan subunit and glycoprotein link, which aggregated to form a protein-polysaccharide complex. Subsequently the aggregation has been shown to require the interaction of hyaluronate and a protein-rich fraction (Gregory, 1973; Hardingham & Muir, 1974; Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974).

The present work represents a search for similar aggregates in the human intervertebral disc.

Materials and Methods

Guanidinium chloride was prepared from guanidinium carbonate (Kawahara & Tanford, 1966). CsCl was the Sequanal grade (Pierce Chemical Co., Rockford, Ill., U.S.A.). All other materials were commercial reagent grade.

Densities were determined gravimetrically by using a 500 μ l Kirk micropipette. Viscosity was measured at $20.0 \pm 0.01^\circ\text{C}$ in a semimicro viscometer with a flow time for water close to 60 s (Model A-100; Cannon Instrument Co., State College, Pa., U.S.A.).

Hexuronate was determined by the method of Bitter & Muir (1962). Absorbance was measured at 280 nm in a Beckman DU spectrophotometer in 10 mm cells with a 9 mm spacer.

Residues were analysed after dissolution for 24 h at 37°C in 5 ml of 0.5M-NaOH/g fresh wt.; the supernatant was sampled after centrifugation. This method produced cleaner dispersions of the residue and results corresponding to those obtained by the H₂SO₄ procedure of Hascall & Sajdera (1969).

Specimens

Human lumbar spines were collected within 24 h of death from males who had died of some acute cause and who had no history either of disease affecting the spine or of a connective-tissue disorder. Those specimens dissected immediately were chilled in ice during transport; those for which dissection was delayed were stored at -20°C and thawed at 4°C . After dissection, the discs were stored at -20°C until extraction.

Scheme 1. Flow chart for the fractionation of tissue proteoglycans

Direct extraction proceeded from point (I) to point (II) without the intervening steps; sequential extraction included all steps. The labels (A)–(G) identify the corresponding fractions on the schlieren diagrams of Figs. 3, 5 and 6 and in Table 2. Details are given in the Materials and Methods section.

Direct extraction

The method used was essentially that of Hascall & Sajdera (1969). All extractants were buffered by the addition of acetic acid to 0.05M; the pH was adjusted to 5.8 with conc. NH_3 . Tissue was ground in a Latapie mill (A. H. Thomas, Philadelphia,

Pa., U.S.A.), suspended in 15ml of extractant/g fresh wt. and mixed gently for 24h at 22°C in a rotary extractor (Sweeny *et al.*, 1963). After centrifugation for 15min at 2000g and 22°C, the supernatant was decanted and clarified with 50mg of Hyflo Supercel/ml. The filter cake was discarded.

Sequential extraction

Ground tissue was treated as described under 'Direct extraction' with buffered 0.4M-guanidinium chloride as the extractant. The residue was re-extracted twice for two 2h periods, and then for a further 24h with buffered 4M-guanidinium chloride.

A simplified flow chart describing the extraction procedures is given in Scheme 1.

Density-gradient ultracentrifugation

The two-stage procedure of Hascall & Sajdera (1969) was followed. A Spinco model L preparative ultracentrifuge with a type 50 head and cellulose nitrate tubes was run for 40–48h at 20°C and 96500g (r_{av} , 5.4cm). After puncture of the bottom of the tube, fractions were collected by displacement of its contents from above with kerosene delivered from a syringe.

Analytical ultracentrifugation

All measurements were made at 20°C in buffered 0.4M-guanidinium chloride in a Spinco model E analytical ultracentrifuge at 167000g (48000rev./min, r_{av} , 6.5cm). Double-sector cells with sapphire windows and 12mm charcoal-filled Epon centre-pieces were used. Timing was begun on reaching full speed. Results were recorded by using schlieren optics and 5cm×25cm Kodak metallographic plates.

Fig. 1. Extraction of proteoglycans by various reagents

The fraction of the proteoglycan extracted was expressed as the percentage of the total hexuronate of the tissue (extract plus residue) found in the extract. —, Inter-vertebral disc; ----, bovine nasal cartilage. KCl, □; urea, △; MgCl₂, ●; guanidinium chloride, ○.

Results and Discussion

Discs from a stillbirth and from two men, aged 23 and 58 years, were used in the following experiments, and gave similar results.

Extraction of disc proteoglycans

The efficiency of extraction from cartilage and disc was examined by using a variety of reagents which had been used by others with the former tissue (Fig. 1). The results with cartilage corresponded to published data (Sajdera & Hascall, 1969; Mason & Mayes, 1973). With the possible exceptions of 8M-urea and 4M-MgCl₂, the amount of hexuronate extracted from disc appeared to be independent of the extractant used. Water and 0.15M-NaCl gave similar yields, confirming the earlier work of Hallén (1970) with fewer extractants.

Since both tissues contain proteoglycans of similar constitution (Rosenberg *et al.*, 1967) embedded in a network of insoluble collagen, the macro-

Fig. 2. Density-gradient centrifugation of 4M-guanidinium chloride extracts in 0.4M-guanidinium chloride, pH 5.8

After centrifugation, 2ml fractions were taken. Density (○), hexuronate (●) and E₂₈₀ (■) were measured on each. The supernatant gel was discarded.

Fig. 3. Ultracentrifugal analysis of density-gradient fractions from 4M-guanidinium chloride extracts

The direction of sedimentation is from left to right. The vertical line represents the solvent-solute meniscus. Bar angle 50° . Photographs were taken 20 min after reaching speed. The labels of the fractions correspond to those in Scheme 1. Concentration is expressed in μmol of hexuronate/ml. The upper row represents extracts of nasal cartilage; the lower row, disc. Bovine nasal cartilage: (D), $2.7 \mu\text{mol/ml}$; (E), $5.2 \mu\text{mol/ml}$; (F), $0.8 \mu\text{mol/ml}$; (G), $4.0 \mu\text{mol/ml}$. Human intervertebral disc: (D), $3.2 \mu\text{mol/ml}$; (E), $2.7 \mu\text{mol/ml}$; (F), $0.7 \mu\text{mol/ml}$; (G), $3.0 \mu\text{mol/ml}$.

molecular organization of the disc appeared to differ from that of cartilage.

Demonstration of proteoglycan complexes

Evidence for the presence of proteoglycan aggregates in disc was sought by using the procedures devised by Hascall & Sajdera (1969) for bovine nasal cartilage. Disc or cartilage was extracted with buffered 4M-guanidinium chloride, the clarified extracts were adjusted to associative conditions by dialysis against 9 vol. of 0.05M-ammonium acetate buffer, pH 5.8, and centrifuged in a CsCl gradient (Fig. 2). With both tissues, a protein-rich fraction rose to the top. The gel that formed at the top of the tube was assumed to comprise collagen. The bottom two fractions were pooled for each tissue and a portion was examined in the analytical ultracentrifuge after dialysis against buffered 0.4M-guanidinium chloride to remove CsCl (Fig. 3, D). The bimodal pattern reported for cartilage by Hascall & Sajdera (1969) was confirmed. The disc extracts also showed a bimodal pattern; the more rapidly sedimenting peak was very much less prominent and separated poorly from the more slowly sedimenting component. The disc appeared to contain a small amount of proteoglycan aggregate similar to that of bovine nasal cartilage.

The possible reversibility of aggregation was examined further by density-gradient centrifugation under dissociative conditions (Hascall & Sajdera, 1969). An equal volume of buffered 7.5M-guanidinium

chloride was added to a portion of fraction D (Scheme 1) and the density adjusted by the addition of CsCl. After centrifugation to equilibrium, both tissues yielded a hexuronate-rich high-density fraction (Fig. 4). Again, the yield with disc was about one-eighth that with cartilage. The two bottom fractions were pooled for each tissue, dialysed against buffered 0.4M-guanidinium chloride, and examined in the analytical ultracentrifuge (Fig. 3, E). As reported by Hascall & Sajdera (1969), cartilage showed a single symmetrical peak sedimenting like the more slowly sedimenting peak of the starting material. Although the results with disc were similar, the peak sedimented more slowly and was broader at a corresponding position in the cell, suggesting that the proteoglycan of disc was more polydisperse and of lower molecular weight than that of cartilage. The low-density fractions remaining in the upper part of the tubes were also dialysed against buffered 0.4M-guanidinium chloride and examined in the analytical ultracentrifuge (Fig. 3, F). No clear peak was seen with either tissue, excluding the possibility that the second peak seen in Fig. 3(D) represented a separate protein-rich

Fig. 4. Density-gradient centrifugation of high-density fractions (D) in 4M-guanidinium chloride

Fractions 1 and 2 (Fig. 2) were pooled and centrifuged in a CsCl gradient; 2 ml fractions were taken and analysed as described in Fig. 2. Symbols are as in Fig. 2.

macromolecular component. A similar pair of tubes was remixed after centrifugation in 4M-guanidinium chloride, dialysed against buffered 0.4M-guanidinium chloride and examined in the analytical ultracentrifuge (Fig. 3, G). Although recombination of the high- and low-density fractions of both tissues resulted in the regeneration of the bimodal pattern, the proportion of the more rapidly sedimenting component was less than in the initial ultracentrifugation (cf. Fig. 3, D). The low-density fraction of disc proteoglycan appeared to be essential for the formation of aggregate, as reported for cartilage (Hascall & Sajdera, 1969); the incompleteness of reversibility has also been reported by this group (Gregory *et al.*, 1970).

In addition, the formation of complex was examined viscometrically (Table 1). Much of the viscosity of the complex of cartilage (fraction D) was lost when it was dissociated into its high- and low-density components, fractions (E) and (F) respectively. Recombination of these components (fraction G) restored most, but not all, of the initial viscosity. With intervertebral disc, the initial viscosity of fraction (D) was much less at equivalent concentration. After dissociation, a much greater viscosity was found in the hexuronate-rich component (fraction E), with little in the protein-rich component (fraction F). Their viscosities were comparable with those of the corresponding fraction of cartilage. Recombination of the components of disc (fraction G) yielded a viscosity intermediate between those of fractions (D) and (E). Since the viscosities associated with the high- and low-density components of disc and of cartilage (fractions E and F respectively) were comparable, the lesser viscosities found in the fractions of disc showing complex-formation (fractions D and G) suggested that a much smaller proportion of the disc proteoglycan was involved in complex-formation, a conclusion conforming to the findings on analytical ultracentrifugation. The cartilage data confirmed that of Hascall & Sajdera (1969).

Considered both as a whole and in relation to the work of Hascall & Sajdera (1969), the data suggest that the two components found by ultracentrifugation

of the disc extract represent a proteoglycan monomer and an aggregate formed by its reversible association with other components of the tissue which had lower density in a CsCl gradient. The complex was more difficult to demonstrate with disc than with cartilage because it represented a much smaller proportion of the total proteoglycan.

Sequential extraction

If dissociation of the complex is necessary for the extraction of aggregated proteoglycan (Gregory *et al.*, 1970), prior extraction with a non-dissociative solvent would seem likely to remove the free proteoglycan from a tissue. Subsequent treatment with a dissociative solvent would then be expected to remove the aggregated proteoglycan selectively. To test this hypothesis, samples of cartilage and disc were extracted consecutively with an associative solvent, buffered 0.4M-guanidinium chloride, and a dissociative solvent, buffered 4M-guanidinium chloride (Scheme 1; Table 2). The quantities of hexuronate extracted from the tissues with 0.4M-guanidinium chloride were comparable. Two washes with this

Table 1. *Viscosities of fractions prepared from 4M-guanidinium chloride extracts by density-gradient centrifugation*

The preparation of the fractions is described in Scheme 1. All measurements were made in 0.4M-guanidinium chloride-0.05M-ammonium acetate, pH 5.8.

Fraction	Hexuronate ($\mu\text{mol/ml}$)	Specific viscosity
Bovine nasal cartilage		
(D)	4.6	2.64
(E)	4.0	0.80
(F)	0.7	0.26
(G)	4.7	1.90
Human intervertebral disc		
(D)	4.3	0.95
(E)	4.6	0.84
(F)	0.8	0.22
(G)	5.4	0.90

Table 2. *Sequential extraction of tissue hexuronate*

Each value represents the mean result from three specimens. Hexuronate is expressed as $\mu\text{mol/g}$ wet wt. and as a percentage of the total hexuronate extracted.

Extract	Human intervertebral disc		Bovine nasal cartilage	
	(μmol of hexuronate/g)	(%)	(μmol of hexuronate/g)	(%)
0.4M-Guanidinium chloride	67.5	85.3	72.9	31.0
Wash 1	4.5	5.7	4.3	1.7
Wash 2	1.3	1.6	1.5	0.6
4M-Guanidinium chloride	5.7	7.4	156.1	66.7
Total	79.0	100.0	234.8	100.0

Fig. 5. Ultracentrifugal analysis of 0.4M-guanidinium chloride extracts and of subsequent 4M-guanidinium chloride extracts

The labels correspond to the fractions described in Scheme 1. Photographs were taken 20 min after reaching speed; bar angle 50°. Concentration is expressed in μmol of hexuronate/ml; upper row, bovine nasal cartilage: (A), 4.3 $\mu\text{mol}/\text{ml}$; (B), 3.2 $\mu\text{mol}/\text{ml}$; (C), 5.4 $\mu\text{mol}/\text{ml}$; (D), 5.9 $\mu\text{mol}/\text{ml}$. Lower row, human intervertebral disc: (A), 4.1 $\mu\text{mol}/\text{ml}$; (B), 0.9 $\mu\text{mol}/\text{ml}$; (C), 4.2 $\mu\text{mol}/\text{ml}$; (D), 1.6 $\mu\text{mol}/\text{ml}$.

extractant removed very little further hexuronate, suggesting that the initial extractions had been essentially complete. Subsequent treatment with 4M-guanidinium chloride extracted 25 times more hexuronate from cartilage than from disc. In general, the ratios of hexuronate in the 0.4M- to that in the 4M-guanidinium chloride extracts corresponded to the relative proportions of the slowly and rapidly sedimenting components of the two tissues, in conformity with the hypothesis.

The fractions were examined in the analytical ultracentrifuge after dialysis against buffered 0.4M-guanidinium chloride (Fig. 5). The 0.4M-guanidinium chloride extract of cartilage (A) showed two components, that which sedimented more rapidly being the more prominent and resembling the non-aggregated proteoglycan of cartilage. The less abundant, more slowly sedimenting component resembled the extraneous protein of Gregory *et al.* (1970). The 4M-guanidinium chloride extract of cartilage (B) contained the same two components in much smaller amount, particularly with respect to the non-aggregated proteoglycan, and an additional substantial sharp peak of material corresponding in ultracentrifugal behaviour to the aggregate. In all these respects, the results agreed with those of Gregory *et al.* (1970).

On the other hand, in the 0.4M-guanidinium chloride extract of disc (A), the more slowly sedimenting component was the more prominent and behaved like the free proteoglycan (Fig. 5). A faster minor component resembled the aggregate seen in disc

(cf. Fig. 3, D). The presence of complex in such extracts has been attributed to diffusible and surface aggregates (Gregory *et al.*, 1970). The 4M-guanidinium chloride extract of disc (B) showed only two components in amounts that were approximately equal but in a too-low concentration to permit clear interpretation.

In an effort to elucidate the composition of the crude extracts, they were dialysed against buffered 0.4M-guanidinium chloride, CsCl was added to a density of 1.50 g/ml and they were centrifuged in a 10 ml tube for 48 h at 86500g and fractionated. The bottom two 2 ml fractions were pooled, dialysed free of CsCl and examined by analytical ultracentrifugation (see Fig. 5 and Scheme 1). The 0.4M-guanidinium chloride extract of cartilage (C) showed a single peak of proteoglycan monomer (Fig. 5); peaks of monomer and of aggregate were seen in the 4M-guanidinium chloride extract (D), corroborating the observations of Gregory *et al.* (1970). The results with disc were similar. The 0.4M-guanidinium chloride extract contained a single component resembling proteoglycan monomer, and the 4M-guanidinium chloride extract (D) showed two components corresponding to monomer and aggregate. Thus, as predicted, prior extraction with 0.4M-guanidinium chloride appeared to have removed most of the proteoglycan monomer from the tissue and to have permitted a clearer demonstration of the presence of aggregate than had been possible with direct extraction. The presence of two components in the 4M-guanidinium chloride extract can be interpreted in various ways: the failure of the first extractant to remove completely the non-aggregated proteoglycan of the tissue; the incomplete reversibility of aggregation, as suggested by Gregory *et al.* (1970); or the denaturation or loss of some component essential for aggregation (Gregory, 1973; Hardingham & Muir, 1972, 1974; Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974).

Further evidence for the presence of both monomeric and aggregated proteoglycan in the 4M-guanidinium chloride extract (fraction B, Scheme 1) was sought by demonstration of reversible dissociation (Fig. 6). To the remainder of the hexuronate-rich fractions (fraction D, Scheme 1) an equal volume of 7.5M-guanidinium chloride was added, the solution ultracentrifuged to equilibrium and the contents of the tube were separated into high-density (lower 40%) and low-density (upper 60%) fractions, as before. After dialysis against buffered 0.4M-guanidinium chloride, the high-density fractions of both cartilage and disc (E) contained a single peak (Fig. 6); the disc proteoglycan sedimented more slowly than that of cartilage and showed a broader peak. No peak was seen with either low-density fraction (F). Re-mixing a replicate tube before dialysis

Fig. 6. Ultracentrifugal analysis of the density-gradient fractions from sequential 4M-guanidinium chloride extracts

The labels correspond to the fractions described in Scheme 1. Photographs were taken 20min after reaching speed; bar angle 50°. Concentration is expressed as μmol of hexuronate/ml. Upper row, bovine nasal cartilage: (E), 5.4 $\mu\text{mol}/\text{ml}$; (F), 0.4 $\mu\text{mol}/\text{ml}$; (G), 4.8 $\mu\text{mol}/\text{ml}$. Lower row, human intervertebral disc: (E), 5.9 $\mu\text{mol}/\text{ml}$; (F), 0.6 $\mu\text{mol}/\text{ml}$; (G), 1.7 $\mu\text{mol}/\text{ml}$.

demonstrated the partial reformation of aggregate (G). Thus the two peaks seen in Fig. 5(D) represented free proteoglycan and aggregate, as expected from previous results.

General discussion

Bovine nasal cartilage has been studied in parallel with the human intervertebral disc throughout this work because the extensive studies of the former tissue provided a well-established basis for comparison. The findings of the Rockefeller group have been reproducible in our hands (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969; Gregory *et al.*, 1970), thus minimizing the possibility that unintentional differences in procedure may have distorted the data for disc.

A proteoglycan aggregate similar to the protein-polysaccharide complex of cartilage appears to occur in disc. However, its small proportion of the total proteoglycan, 5–10% rather than the 40–60% reported for cartilage (Hascall & Sajdera, 1969), made the demonstration of its presence uncertain. The sequential-extraction procedure used by Gregory *et al.* (1970) was useful in resolving this difficulty.

Proteoglycans of bovine nasal cartilage, prepared by fractionation of high-shear homogenates (Rosenberg *et al.*, 1970), have been compared with proteoglycans prepared by the methods used in the present work. Their fractions PPL5 showed a bimodal schlieren pattern similar to the aggregated proteoglycan, and fraction PPL3 a unimodal pattern similar to our non-aggregated proteoglycan. Proteoglycans of bovine intervertebral disc, similarly prepared by salt fractionation of high-speed homogenates and analysed

in the ultracentrifuge (Rosenberg *et al.*, 1967), produced a PPL5 fraction with a bimodal pattern resembling that shown by our 4M-guanidinium chloride extract. Their PPL3 fraction showed a unimodal pattern resembling that shown by our 4M-guanidinium chloride extract. Thus the correspondence between these two fractions appears to extend from cartilage to disc.

The incomplete reversibility of the disaggregation has been a concern in the interpretation of our results. The earlier papers of the Rockefeller group appear to imply complete reversibility (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). Although reversibility appears not to have been studied directly, subsequent work has revealed an unexpected complexity to aggregation (Gregory, 1973; Hardingham & Muir, 1972, 1974; Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974). The possibility of loss of an essential component was avoided by remixing the tubes in which the components had been separated rather than by cutting and recombining fractions; the latter approach was found to aggravate the problem. Remixing should also have eliminated possible changes in the relative proportions of the various components of the system. A more likely explanation of the incomplete reversibility is the degradation or denaturation of some component.

The supportive function of the intervertebral disc appears to depend on the constitution and integrity of its component proteoglycans (Lyons *et al.*, 1966; Gower & Pedrini, 1969), in much the same manner as in hyaline cartilage (Gregory *et al.*, 1970). However, these data suggest that aggregates themselves are unlikely to make an important contribution to the mechanical behaviour of disc.

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