A Method for the Determination of Hyaluronate in the Presence of Other Glycosaminoglycans and its Application to Human Intervertebral Disc

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A highly sensitive proteoglycan-binding assay was developed for the detection and determination of small amounts of hyaluronate. Application of this assay to human intervertebral-disc tissue showed that the radial distribution of hyaluronate closely followed the distribution of proteoglycans.

Hyaluronate is a long-chain unbranched polysaccharide consisting of a repeating disaccharide unit (GlcAβ1–3GlcNAc) β1–4-linked, which has a broad distribution in vertebrate connective tissue (Laurent, 1970). It is present in cartilage in only small amounts, but it was shown to be an important component in the macromolecular organization of cartilage proteoglycans (Hardingham & Muir, 1972, 1973, 1974; Hascall & Heinegård, 1974). Each hyaluronate chain forms the backbone of multiple aggregates of chondroitin sulphate proteoglycans.

The invertebral disc has been broadly classified as a cartilaginous tissue, and in common with cartilage it has a high collagen and glycosaminoglycan content. However, the proteoglycans have been shown to differ considerably in composition from those found normally in cartilage, as they contain much more keratan sulphate and protein (Heinegård & Gardell, 1967; Gower & Pedrini, 1969). Hyaluronate has been identified as one of the glycosaminoglycans present and may thus participate in the organization of the proteoglycans (Antonopoulos, 1965; Solheim, 1966). The present study was undertaken to confirm the presence of hyaluronate in the disc and to determine its distribution in different parts of the tissue before investigating its possible role in the macromolecular organization of the proteoglycans.

There is no simple method for the rapid detection and determination of small amounts of hyaluronate. The methods available largely depend on its isolation and chemical characterization, such as by using cetylpyridinium chloride–cellulose micro-columns (Antonopoulos *et al.*, 1961) or by a combination of methods, such as cellulose acetate electrophoresis (Rodén *et al.*, 1972) and enzymic digestion with testicular hyaluronidase (EC 3.2.1.35) or the more specific leech hyaluronidase (Brimacombe & Webber, 1964). These methods are satisfactory, but are made considerably more difficult if there is a large excess of other glycosaminoglycans present in the sample being examined.

The binding of cartilage proteoglycans to hyaluronate in the formation of high-molecular-weight aggregates and the ability to separate them by gel chromatography (Hardingham & Muir, 1972, 1974) appeared to form the basis of a sensitive and highly specific assay that could be applied to relatively impure mixtures of glycosaminoglycans. The only assay of comparable sensitivity and specificity is that of Laurent *et al.* (1969). This is a very accurate isotopic-dilution method which requires, however, the preparation of ^{14}C-labelled hyaluronate and a specific bacterial hyaluronate lyase (EC 4.2.2.1) and involves a large number of procedures in each assay. The proteoglycan-binding assay reported here is of comparable sensitivity with the isotopic-dilution method and requires many fewer manipulations in each assay.

Experimental

All reagents and analytical methods were as described in Hardingham & Muir (1974). Standard hyaluronate (sodium salt) and heparin were obtained from BDH Chemicals, Poole, Dorset, U.K. Chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate were obtained from Seikagaku Kogyo Co., Tokyo, Japan. Corneal keratan sulphate (KS-I) and skeletal keratan sulphate (KS-II) were standards supplied by Dr. M. B. Mathews, Dr. J. A. Cifonelli and Dr. L. Rodén, University of Chicago, Chicago, IL, U.S.A.

Post-mortem tissues for analysis were obtained from the L_5/S_1 disc from a human female aged 65 years and the L_3/L_4 disc of a human male aged 5 years. Samples were dissected free of adhering tissue and serial sections were cut from the outer anterior region of the annulus fibrosus (sample 1) to the inner edge (sample 4) and a random sample of nucleus pulposus was taken (sample 5). Samples were kept frozen at $-20°C$ before analysis.

Tissue samples were dried and then digested with crystalline papain (10μl of enzyme suspension/

200 mg of tissue per ml of buffer) as described by Adams & Muir (1976) The digest was dialysed against water for 48 h in heated Visking tubing (Callanan *et al.*, 1957), and the uronic acid content and molar ratio glucosamine/galactosamine determined. For the assay of hyaluronate the glycosaminoglycans were precipitated with cetylpyridinium chloride and converted into sodium salts as described by Hardingham & Muir (1974).

Assay of hyaluronate

Cartilage proteoglycans were prepared from pig laryngeal cartilage by CsCl-density-gradient centrifugation under associative and dissociative conditions as described by Hardingham & Muir (1974); 10 g of wet cartilage yielded sufficient proteoglycan for more than 100 assays.

Samples of glycosaminoglycans isolated from papain digests were dissolved in distilled water and heated in a boiling-water bath for 30 min to inactivate residual proteolytic activity. The solutions were analysed for uronic acid, and a sample containing 1–20 μg of hyaluronate was added to a standard amount of disaggregated cartilage proteoglycan which contained 1.0 mg of uronic acid in 0.5 M-guanidinium chloride/0.05 M-sodium acetate, pH 6.8. As it was found to be more accurate, both the amount of sample taken and the proteoglycan to which it was added were measured by weight. The final volume was made to 1.0 ml with 0.5 M-guanidinium hydrochloride/0.05 M-sodium acetate, pH 6.8, and the solution was applied to a column (165 cm × 1.1 cm) of Sepharose 2B eluted upwards with 0.5 M-sodium acetate, pH 6.8, (6 ml/h) at 4°C. Fractions (2.5 ml) were collected, and their uronic acid contents measured. The proportion of total uronic eluted in the regions of the void volume was estimated by cutting and weighing traced elution profiles. Mixtures of a constant amount of disaggregated proteoglycan with various amounts of hyaluronate were chromatographed to produce a standard curve. The effect of other glycosaminoglycans on the position of elution of disaggregated proteoglycan and on the binding of hyaluronate to disaggregated proteoglycan was also measured.

Cetylpyridinium chloride-cellulose column chromatography

Cetylpyridinium chloride-cellulose column chromatography was carried out essentially as described by Antonopoulos *et al.* (1961). A column (11.0 cm × 1.1 cm) was packed with defined degassed cellulose, Whatman CF11 (H. Reeve Angel, London E.C.4, U.K.) in 1% (w/v) cetylpyridinium chloride. Glycosaminoglycans from human intervertebral disc (10 mg) in 0.5 ml of 5 mM-Na₂SO₄ were applied to the column, which was then eluted stepwise with 3 × 4 ml of 1% cetylpyridinium chloride, 4 × 4 ml of 0.3 M-NaCl, 4 × 4 ml of 0.2 M-MgCl₂, 4 × 4 ml of 0.4 M-MgCl₂, 4 × 4 ml of 0.6 M-MgCl₂ and 4 × 4 ml of 2 M-HCl. All the salt solutions contained 0.05% cetylpyridinium chloride. Fractions (4 ml) were collected, and their uronic acid contents determined. The positions of elution of standard hyaluronate and chrondroitin sulphate were also tested on the column.

Results

Gel chromatography of mixtures of disaggregated proteoglycan with increasing amounts of hyaluronate showed a linear increase in the proportion of uronic acid excluded from the gel with up to 12 μg of hyaluronate added to 4.0 mg of proteoglycan, i.e. a proteoglycan/hyaluronate ratio of 333:1 (w/w) (Fig. 1). With between 15 and 300 μg of hyaluronate in the mixture, binding was saturated, as the proportion excluded from the gel was constant (about 53%). With more than 300 μg of hyaluronate the proportion excluded decreased, and the excluded peak was not resolved well from the unbound proteoglycan.

The effect of papain digestion and boiling on hyaluronate was assessed by gel chromatography on Sepharose 2B (Fig. 2). Although papain digestion resulted in some decrease in size, it did not affect the ability of the hyaluronate to bind to proteoglycan. Boiling for 30 min gave no further decrease in the size of papain-digested hyaluronate or any decrease in its binding to proteoglycan. The procedure adopted therefore did not affect the ability of the hyaluronate
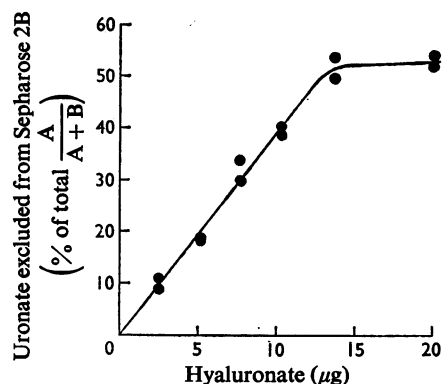

Fig. 1. *Calibration curve of binding of disaggregated cartilage proteoglycans to various amounts of hyaluronate*

Samples were mixed and fractionated on a column of Sepharose 2B as described in the text. Areas A and B are shown in Fig. 2.
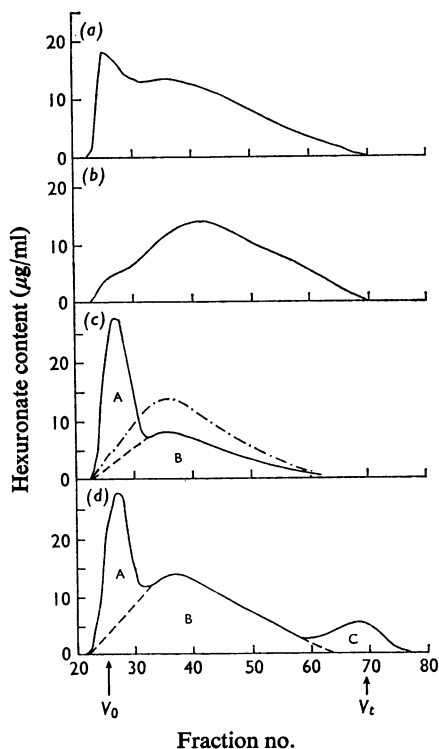
Fig. 2. *Gel chromatography on Sepharose 2B of standard hyaluronate and proteoglycan/hyaluronate mixtures*

(a) Standard hyaluronate and (b) hyaluronate after digestion with papain as described in the text; (c) dis-aggregated cartilage proteoglycan with (——) and without (–·–·) added hyaluronate; (d) disaggregated proteoglycan mixed with glycosaminoglycans containing some hyaluronate. The proportion of uronate excluded from the gel in (c) and (d) was calculated from the areas A/A+B. Area C corresponded to the non-binding glycosaminoglycans and was omitted from the calculation.

to bind proteoglycans. Other experiments have shown that it would have to be decreased extensively to 30000–40000 mol.wt. before it would fail to give a clearly resolved excluded peak on Sepharose 2B when bound to proteoglycan (T. E. Hardingham, unpublished work).

Standard chondroitin 4-sulphate, chondroitin 6-sulphate, corneal keratan sulphate (KS-I), skeletal keratan sulphate (KS-II), dermatan sulphate or heparin (2 mg in each case) were mixed with 4.0 mg of cartilage proteoglycan and chromatographed on Sepharose 2B in the presence or absence of 15 μg of hyaluronate. None interfered with the binding of the hyaluronate to proteoglycan, but both dermatan sulphate and skeletal keratan sulphate produced a

small excluded peak in the absence of added hyaluronate. This effect was removed by pre-digesting the sample with testicular hyaluronidase (EC 3.2.1.35; Boehringer, Mannheim, W. Germany), which suggested that these standards were contaminated with a trace of hyaluronate, 0.3 % (w/w) in dermatan sulphate and 0.8 % (w/w) in skeletal keratan sulphate.

The chromatography was not significantly affected by applying the sample in 0.5–1.5 ml of solution, but the application of a solution at 30°C to the column slightly decreased the proportion of uronic acid excluded from the column. The samples were thus allowed to equilibrate at 4°C for at least 1 h before chromatography. The most likely sources of error in the method are in obtaining good resolution of the free and bound proteogycans by gel chromatography and in the determination of the areas under the elution profiles. It was helpful to adopt a fixed routine in these procedures to obtain consistent results, and with these precautions the method gave a standard deviation ±7.1 %.

The proteoglycan-binding assay was applied to the papain digests of tissue samples from human inter-vertebral disc to determine the total content of hyaluronate in the nucleus pulposus and its radial distribution in the annulus fibrosus. The contents of chondroitin sulphate and keratan sulphate reflecting the distribution of proteoglycans were calculated from uronic acid and glucosamine/galactosamine molar ratios of the same fractions, (Table 1), assuming regular repeating disaccharide structures of these glycosaminoglycans. It was previously shown that there was a steady change in composition across the annuli fibrosi of all lumbar discs of a 44-year-old human spine; the collagen content decreased and uronic acid increased from the outer to inner layers (Adams & Muir, 1976). There was also an increase in type II collagen relative to type I from the outer to inner region of the human disc (Eyre & Muir, 1976). The content of glycosaminoglycan in both the 65-year-old and 5-year-old discs also showed radial changes across the disc. Chondroitin sulphate increased in parallel with water content from the outer to the inner zone of the annulus fibrosus, and its content was highest in the inner annulus fibrosus and nucleus pulposus fractions. The ratio of keratan sulphate/chondroitin sulphate in the old disc was about three times that of the young disc. However, in both it showed a radial distribution similar to chondroitin sulphate, but the increase inwards was less marked and it thus accounted for less of the total glycosaminoglycans in nucleus pulposus than in outer annulus fibrosus (Table 1).

There was a significant amount of hyaluronate present in all the samples analysed and it represented from 1.8 % of the total tissue uronic acid in the young nucleus pulposus to 12 % in old human annulus fibrosus. Except in the young nucleus pulposus

Table 1. *Analysis of hyaluronate and other glycosaminoglycans in papain digests of radial sections of two human intervertebral discs*

The total glycosaminoglycan content was calculated as described in the text. A.F., annulus fibrosus; N.P., nucleus pulposus.

Sample	Total glycosaminoglycan (% dry wt. of tissue)	Molar ratio $\dfrac{GlcN}{GalN}$	Hyaluronate	
			(% of total glycosamino-glycan)	(% wet wt. of tissue)
5-year-old L_3/L_4 disc				
A.F. 1	10.49	0.50	2.86	0.115
2	12.11	0.55	3.54	0.098
3	24.10	0.54	3.03	0.167
4	31.65	0.39	3.35	0.197
N.P. 5	33.08	0.31	1.14	0.055
65 year-old L_5/S_1 disc				
A.F. 1	8.20	1.73	4.21	0.157
2	11.30	1.65	3.74	0.172
3	15.84	1.55	4.17	0.252
4	25.64	1.15	4.00	0.327
N.P. 5	24.27	1.14	3.80	0.273

where the content was low the hyaluronate distribution closely followed the distribution of proteoglycans (Table 1) and was similar to that suggested by the results of Szirmai (1970)

As an independent method of assay, hyaluronate was also separated from other glycosaminoglycans from a tissue sample of annulus fibrosus by cetylpyridinium chloride-cellulose column chromatography. The hyaluronate fraction, soluble in 0.3 M-NaCl, accounted for 10.0% of the uronic acid in the sample, and hexosamine analysis showed it to be 87% glucosamine. The fraction bound strongly to proteoglycans, whereas the other fractions eluted with cetylpyridinium chloride, 0.2 M-MgCl$_2$, 0.4 M-MgCl$_2$ and 0.6 M-MgCl$_2$ showed very little binding. The proteoglycan-binding assay showed the hyaluronate content to be 10.7% of the uronic acid and the column chromatography was thus in reasonable agreement with it.

Discussion

Cartilage proteoglycans bind specifically to hyaluronate, and previous results of gel chromatography, viscometry and electron microscopy (Hardingham & Muir, 1972, 1974; Hascall & Heinegård, 1974; Rosenberg et al., 1975) have shown that each proteoglycan possesses a single specific binding site on its protein core. Under conditions of maximum binding the proteoglycans pack next to each other on the hyaluronate chain and were calculated to be about 24 nm apart, i.e. they were occupying a length of hyaluronate of about 10 000 mol.wt. (Hardingham & Muir, 1973). Thus hyaluronate of 5×10^5 mol.wt. can bind up to 100 proteoglycan molecules per chain.

The binding of proteoglycan to hyaluronate is an equilibrium in which complex-formation is favoured at 4°C and at neutral pH (Hardingham & Muir, 1972, 1974), and under these conditions it is largely possible to separate free and bound proteoglycans by gel chromatography. The rate of exchange between free and bound proteoglycans must be quite low as it was also possible to separate them in 0.5 M-guanidinium chloride, pH 5.8, at 20°C in the ultracentrifuge, even though the complex was more dissociated under these conditions (T. E. Hardingham, unpublished work).

It has yet to be determined how far hyaluronate is involved in the organization of the proteoglycans of the disc. Only a very small amount of proteoglycan aggregate was reported in human invertebral disc (Emes & Pearce, 1975). The hyaluronate content of disc reported here was higher than that found in hyaline cartilage from ox or pig, where in nasal or laryngeal cartilage it accounts for less than 1% of the uronic acid (Hardingham & Muir, 1974; Hascall & Heinegård, 1974), but as pig intervertebral disc also had a high content (P. Adams, unpublished work) this was not just a species difference. However, in estimating the stoicheiometry of aggregation it must be remembered that in hyaline cartilage the proteoglycans are of very high molecular weight (1×10^6–4×10^6) and saturation binding between proteoglycan and hyaluronate thus occurs at about 0.7% (w/w) hyaluronate in the mixture, whereas in the intervertebral disc the proteoglycans are of much smaller size (Hopwood & Robinson, 1975) and saturation binding would therefore be at a higher hyaluronate content. Further results have shown that some proteoglycans from human annulus fibrosus and nucleus pulposus are able to bind to hyaluronate.

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