Biochimica et Biophysica Acta, 673 (1981) 443—453

BBA 29557

BIOCHEMICAL CHANGES IN INTERVERTEBRAL DISC DEGENERATION

GILLIAN LYONS *, S. M. EISENSTEIN and M.B.E. SWEET

Departments of Orthopaedic Surgery and Biochemistry, University of the Witwatersrand, Johannesburg 2001 (Republic of South Africa)

(Received September 8th, 1980)
(Revised manuscript received December 5th, 1980)

Key words: Collagen; Proteoglycan; Intervertebral disc

Summary

The distribution of the principal matrix components, collagen, proteoglycans and water, across the diameter of human normal and degenerate intervertebral discs was compared. Little difference in collagen distribution was noted between normal and degenerate tissue but water and proteoglycan content decreased with degeneration, particularly in the centre of the disc.

Proteoglycans of the nucleus pulposus and annulus fibrosus of normal and degenerate intervertebral discs were examined. In comparison with monomers of normal tissue, degenerate disc proteoglycans were of larger average hydrodynamic size and had a higher glucosamine to galactosamine ratio. Proteoglycans were digested with chondroitinase ABC and passed over an HA-Sepharose 2B affinity column. A greater proportion of the keratan sulphate-protein cores from degenerate disc were capable of interaction with the immobilized hyaluronate. Loss of aggregating ability was associated with diminution in size of the core. It is suggested that a large proportion of proteoglycans from normal disc have undergone a degree of degradation in the hyaluronate binding region and that proteoglycan synthesis in this tissue is slower than in degenerate tissue.

Introduction

The intervertebral disc is a connective-tissue structure which links adjacent vertebrae. It is specially adapted to withstand applied load while permitting spinal flexibility [1]. Certain clinical disorders of the lumbar spine arise as a consequence of intervertebral disc degeneration [2—4].

* To whom correspondence should be addressed.

The disc consists of a central nucleus pulposus surrounded by concentric, fibrous layers of the annulus fibrosus. In both nucleus and annulus low cell populations [5] and collagen fibres lie embedded in a gel of proteoglycan and water [6]. The mechanical properties of disc are determined by the nature of the extracellular matrix [7—10]; mechanical failure may result from local variations in chemical composition [11].

After the age of 30, gradual changes in the chemical composition, texture and histology of lumbar discs of man become evident [6,12]. It has been suggested that degeneration of the intervertebral disc represents a form of accelerated aging [13]. The aim of this study was to examine this hypothesis from a biochemical standpoint.

Experimental procedures

Materials. All reagents were of analytical grade with the exception of glucosamine hydrochloride, galactosamine hydrochloride, glucuronolactone, *N*-acetyl neuraminic acid, carbazole and guanidinium chloride. Sepharose 2B, 4B and 6B and Blue Dextran were from Pharmacia, Uppsala, Sweden and BioGel P2 from BioRad Laboratories, Richmond, CA. Whatman DE-52 cellulose was supplied by Whatman Ltd., Springfield Mill, Maidstone, Kent. Umbilical cord hyaluronate (Grade I) was purchased from Sigma Chemical Co.

Analytical methods. Uronic acid was measured by an automated modification [14] of the carbazole-borosulphuric acid method [15] using glucuronolactone as standard. Hydroxyproline was determined by an automated modification [16] of the method of Woessner [17] after hydrolysis in 6 M HCl at 100°C for 24 h in sealed glass tubes under nitrogen. Glucosamine and galactosamine were quantitatively determined on a 20 cm column of Beckman M81 resin on a Model 116 amino acid analyzer. Samples were hydrolyzed in 4 M HCl at 100°C in sealed glass tubes under nitrogen for 4 h. Sialic acid was determined by the method of Jourdian et al. [18] using *N*-acetyl neuraminic acid as standard.

Tissue. Intervertebral disc tissue was obtained from two sources: (a) degenerate material was removed from the lumbar intervertebral spaces of three male patients (aged 39, 50 and 57 years) undergoing anterior spinal fusion surgery for degenerative disc disease. (b) normal tissue was removed similarly from cadavers of three male patients (aged 23, 23 and 25 years) who had already donated their kidneys following the diagnosis of brain death. Upon removal, disc material was immediately frozen in liquid nitrogen and stored at —70°C until used.

After the age of 30, gradual changes in the chemical composition, texture and histology of lumbar discs of man become evident [6,12]. The discs of 23 and 25 year old patients were, therefore, regarded as normal since tissue degeneration due to aging had not yet begun. None of these patients was known to have suffered any spinal disorder.

Dissection and determination of matrix component distribution. Nucleus pulposus and ten individual lamellae of the annulus fibrosus (lamalla 1 being the first well-defined lamella adjacent to the nucleus pulposus; lamella 10, the lamella at the disc periphery) were dissected from the disc at room tempera-

ture. Representative samples of nucleus and lamella 1 to 10 were used for each of the following determinations.

(1). Water content: discs were dissected in a sealed chamber in which humidity was maintained at 100% [19]. Tissue pieces were weighed in tightly stoppered plastic cups and then desiccated at 67° C to a constant dry weight.

(2). Swelling pressure: tissue was swollen to a constant weight in 0.15 M NaCl solution at 4°C [20] followed by desiccation at 67°C to a constant dry weight.

(3). Collagen content: hydroxyproline content of tissue of known weight was measured. Collagen content was calculated by multiplying the hydroxyproline content by 7.4 [21].

(4). Uronic acid content: tissue of known weight was digested with twice crystallized papain (0.1 mg/100 mg dry tissue) in 0.1 M sodium acetate (pH 5.5) containing 5 mM L-cysteine and 0.05 M EDTA for 24 h at 60°C [22]. Protein material was precipitated by the addition of trichloroacetic acid (to 5% w/v) and removed by centrifugation. Glycosaminoglycans were precipitated by addition of 3 vol. of ethanol. The precipitate was reconstituted in 1 ml water and the uronic acid content determined.

Preparative methods

Extraction of proteoglycans. Nucleus pulposus was separated from annulus fibrosus. Annulus immediately adjacent to the nucleus was discarded. Proteoglycans were extracted from small pieces of nucleus and annulus by stirring for 48 h at 2°C in 10 vol. of 4 M guanidinium chloride in 0.05 M sodium acetate (pH 5.8) containing the following proteolytic enzyme inhibitors: 0.01 M Na_2-EDTA/0.005 M benzamidine hydrochloride/0.1 M 6-amino-hexanoic acid and 1 mg per l soya bean trypsin inhibitor [23]. The extracts were filtered through glass wool and residual tissue was washed 3-times with 20 ml water. Combined extracts and washes were dialysed and lyophilised. 94% of the uronic acid-containing material was extracted from the nucleus pulposus by this method and between 83% and 89% was extracted from the annulus fibrosus.

Proteoglycan monomers were purified by ion-exchange chromatography on Whatman DE-52 DEAE-cellulose [24]. Most of the proteoglycans were eluted with 2 M NaCl in 8 M urea. This fraction was dialysed and lyophilised.

Analysis of proteoglycans. Following lyophilisation, crude proteoglycan extracted from tissue with 4 M guanidinium chloride, was chromatographed on an analytical column of Sepharose 2B as described below. Proteoglycan monomer prepared by ion-exchange chromatography was assessed on the same analytical column of Sepharose 2B.

Chondroitin sulphate chains were released from proteoglycan monomers by treatment with 0.05 M NaOH in 1 M sodium borohydride at 45°C for 48 h under nitrogen [25,26]. The reaction was stopped by the dropwise addition of acetic acid (conc.) following which, samples were lyophilised, desalted on Bio-Gel P2, lyophilised and applied to an analytical column of Sepharose 6B as described below.

Proteoglycan monomer was digested with chondroitinase ABC (0.05 U/mg proteoglycan) in 0.1 M Tris-HCl/0.1 M sodium acetate (pH 7.3) at 37°C for 5 h [27]. Digests were cooled in ice and applied to a column of Sepharose-HA at

4°C as described below. Non-aggregating core molecules were washed from the column by 0.5 M guanidinium chloride; those that were capable of interaction with the immobilized hyaluronate eluted in the 4 M guanidinium chloride fraction. Material eluted in the two fractions was collected, dialysed, lyophilised and chromatographed on an analytical column of Sepharose 4B.

Gel chromatography. Analytical columns of Sepharose 2B and 4B (0.6 × 150 cm) were eluted with 0.5 M sodium acetate (pH 6.8) at 1—2 ml per h at 18°C. An analytical column of Sepharose 6B (0.6 × 150 cm) was eluted with 0.2 M NaCl at 1—2 ml per h at 18°C. Fractions of 0.7 ml were collected on fraction collectors equipped with drop counters. All columns were calibrated with proteoglycan aggregates and glucuronolactone. Column effluents were analysed for uronic acid.

Desalting columns of BioGel P2 (1.5 × 25 cm) were eluted with 0.5 M pyridine acetate (pH 7). Columns were calibrated with Blue Dextran and LiCl.

Sepharose-HA columns were prepared according to the method of Christner et al. [28] and its efficiency assessed by chromatography of chondroitinase-digested proteoglycan from bovine nasal cartilage. Results obtained were in agreement with published values. The columns (1 × 15 cm) were eluted with 60 ml of 0.5 M guanidinium chloride/0.05 M sodium acetate (pH 5.8) followed by 60 ml of 4 M guanidinium chloride/0.05 M sodium acetate (pH 5.8) at 4°C. The effluent was monitored for uronic acid using a manual carbazole method [15].

Results

The distribution of the major matrix components across the diameter of the disc was examined in human normal and degenerate material.

In normal disc, water content fell gradually from the nucleus pulposus in the centre of the disc towards the disc periphery (Fig. 1A). Water content of the degenerate nucleus was lower than that of the normal nucleus but was similar at the periphery of both normal and degenerate discs. The water gradient across the diameter of the normal disc became less steep with degeneration.

A similar trend in water content was observed after the tissue was swollen in 0.15 M NaCl (Fig. 1B): there was a steady decrease in hydration from the centre towards the periphery of normal and degenerate discs but the water gradient across the swollen normal disc was steeper. Both normal and degenerate disc tissue became superhydrated when soaked in physiological saline; water content in both increased by between 12 and 16%.

Variation in collagen content was similar in normal and degenerate discs, increasing gradually from the centre towards the periphery (Fig. 1C). In agreement with Naylor et al. [29] there is a slight increase in nuclear collagen and a decrease in collagen content of the mid-annulus of the degenerate disc.

In the normal disc there was little change in the concentration of uronic acid across the nucleus pulposus and inner annulus, but it decreased markedly towards the outer annulus (Fig. 1D). The concentration of uronic acid was lower in the degenerate nucleus and annulus than in normal tissue and showed little variation across the disc diameter.

In contrast to collagen distribution across normal and degenerate discs, obvi-

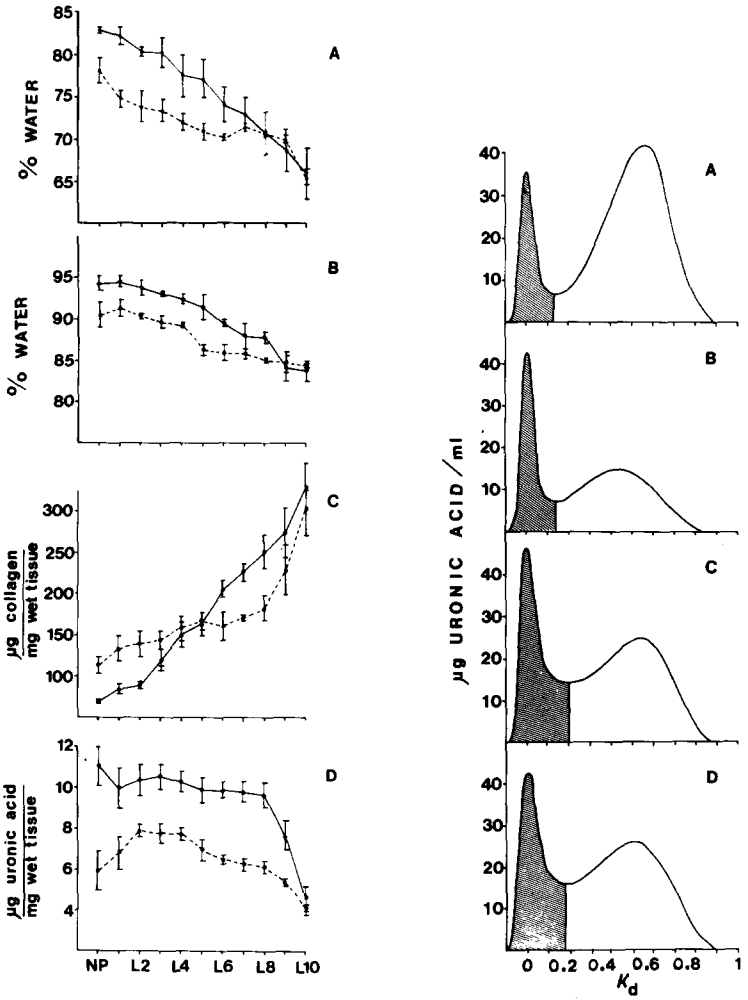

Fig. 1. Distribution of major matrix components across the diameter of human normal (———) and degenerate (- - - - -) lumbar intervertebral discs. Nucleus pulposus (NP) and ten lamellae of the annulus fibrosus (1—10) were dissected from each disc. Water content (A), swelling pressure (B), collagen content (C) and uronic acid content (D) of each sample was determined as described in the text.

Fig. 2. Gel filtration of crude proteoglycans (see text for details) from normal nucleus pulposus (A), normal annulus fibrosus (B), degenerate nucleus pulposus (C) and degenerate annulus fibrosus (D) on an analytical column of Sepharose 2B. Column affluents were analysed for uronic acid. The percentage of uronic-acid positive material excluded from the column (∞) was:- normal nucleus pulposus 20%; normal annulus fibrosus 41%; degenerate nucleus pulposus 36%; degenerate annulus fibrosus 38%.

ous differences in uronic acid concentration (reflecting proteoglycan distribution [11]) and water content were noted between the two tissues. Like proteoglycans of cartilage, those of intervertebral disc are composed of a protein core to which chondroitin sulphate and keratan sulphate are covalently attached at their reducing ends [20]. The protein core has three regions: one rich in chondroitin sulphate, another rich in keratan sulphate and a third lacking glycosaminoglycans [31]. The latter region of the core protein is responsible for the

interaction of proteoglycan with hyaluronate to form large, multimolecular aggregates [32,33]. Proteoglycans enable tissue to withstand compression and, in addition, play an important role in determining the transport of compounds including salts, water, hormones, waste products and gases through the tissue [34—36]. Proteoglycans of nucleus pulposus and annulus fibrosus of normal and degenerate intervertebral discs were thus compared with a view to explaining the differences in hydration of these tissues.

Crude reassociated proteoglycan was chromatographed on Sepharose 2B (Fig. 2) as part of a preliminary set of experiments on small quantities of tissue, in order to estimate the degree of association of proteoglycan with other matrix components. The results were not regarded as definitive because of the possibility of some proteolytic degradation during preparation. With this reser-

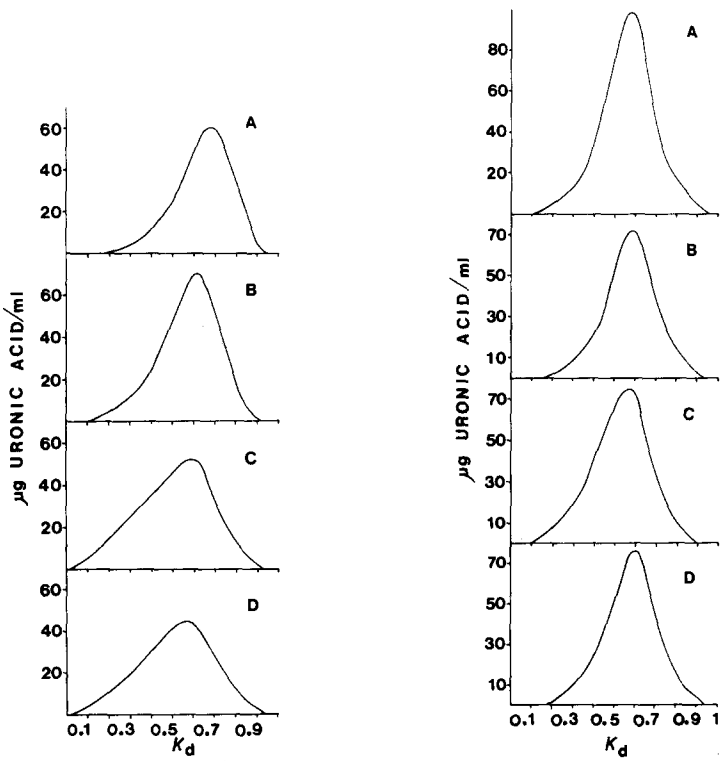

Fig. 3. Gel filtration of proteoglycan monomer on Sepharose 2B. Purified proteoglycan monomer from normal nucleus pulposus (A), normal annulus fibrosus (B), degenerate nucleus pulposus (C) and degenerate annulus fibrosus (D) were chromatographed on Sepharose 2B (0.6 × 150 cm). Column effluents were analysed for uronic acid. The K_d values were: normal nucleus pulposus 0.68; normal annulus fibrosus 0.62; degenerate nucleus pulposus 0.58; degenerate annulus fibrosus 0.56.

Fig. 4. Gel filtration of chondroitin sulphate chains on Sepharose 6B (0.6 × 150 cm). Chondroitin sulphate chains were liberated from proteoglycans of normal nucleus pulposus (A), normal annulus fibrosus (B), degenerate nucleus pulposus (C) and degenerate annulus fibrosus (D) by alkaline borohydride treatment and chromatographed on Sepharose 6B. Column effluents were analysed for uronic acid. The average molecular weights of the chondroitin sulphate chains were calculated to be: normal nucleus pulposus 13 200 (K_d 0.58); normal annulus fibrosus 12 300 (K_d 0.59); degenerate nucleus pulposus 13 200 (K_d 0.58); degenerate annulus fibrosus 11 200 (K_d 0.61) [38].

vation, only 20% of the material extracted from normal nucleus pulposus was excluded from the gel, while much greater proportions of the extracts from normal annulus and from degenerate nucleus and annulus were excluded from the gel (41%, 36% and 38% respectively). The above remarks notwithstanding, the figures given compare favourably with published values [31,37].

Monomeric proteoglycans prepared from crude extract by ion-exchange chromatography were assessed on Sepharose 2B (Fig. 3). Nucleus proteoglycans were of smaller average hydrodynamic size than annulus proteoglycans in normal and degenerate discs. By comparison with proteoglycans from degenerate tissue, normal tissue molecules were more retarded by Sepharose 2B and had a lower glucosamine to galactosamine ratio (Table I).

Chondroitin sulphate side chains, prepared by alkaline borohydride degradation of purified monomer from different regions of disc, were assessed on Sepharose 6B (Fig. 4). The results show little difference in the sizes of the chondroitin sulphate, indicating molecular weights of 13 200 for normal nucleus, 12 300 for normal annulus, 13 200 for degenerate nucleus and 11 200 for degenerate annulus, as calculated from the data of Wasteson [38]. Bushell et al. [39] have reported the average molecular weight of disc chondroitin sulphate to be in the range 12 000—20 000.

Chondroitinase ABC-digested proteoglycan was passed over a Sepharose-HA affinity column to assess the degree of interaction with hyaluronate. Keratan sulphate-protein core not bound to the immobilized hyaluronate was washed from the column with 0.5 M guanidinium chloride. Aggregated core was eluted with 4 M guanidinium chloride (Fig. 5). A greater proportion of annulus core proteins bound to the affinity column as compared with those of the nucleus of both normal and degenerate intervertebral disc. In addition the proportion of proteoglycans capable of aggregation was greater in degenerate than normal tissue.

Following chromatography on the Sepharose-HA affinity column, aggregating (4 M guanidinium chloride fraction) and non-aggregating (0.5 M guanidinium chloride fraction) protein cores were recovered and further assessed by chromatography on Sepharose 4B (Fig. 6). The results indicate that the cores capable of interaction with hyaluronate were larger than those incapable of such interaction.

TABLE I

GLUCOSAMINE TO GALACTOSAMINE RATIO OF PROTEOGLYCAN MONOMER FROM HUMAN NORMAL AND DEGENERATE INTERVERTEBRAL DISC

Sample	Ratio (μg)
Normal nucleus pulposus	0.4
Normal annulus fibrosus	0.56
Degenerate nucleus pulposus	0.63
Degenerate annulus fibrosus	0.63

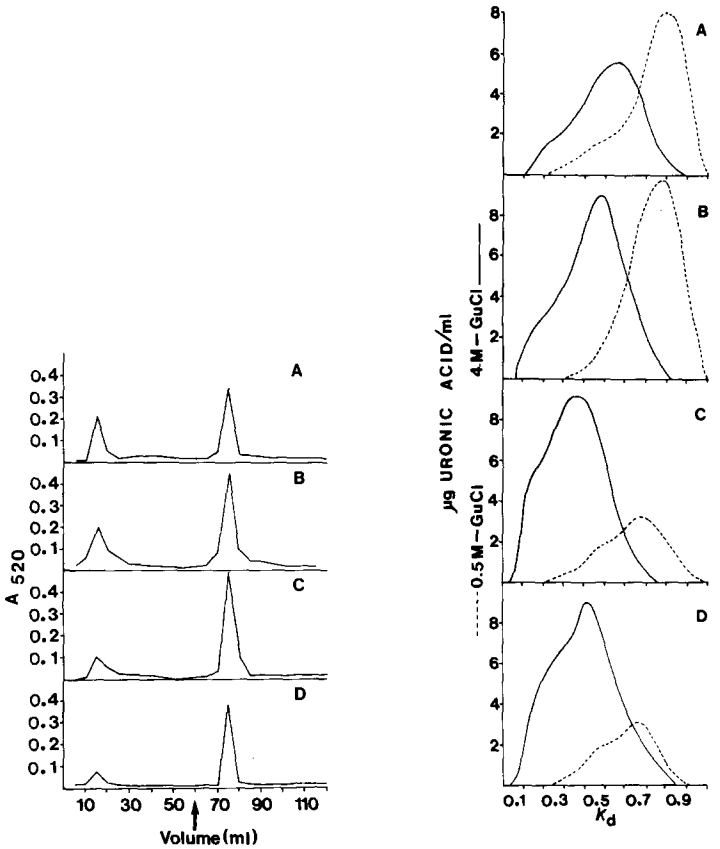

Fig. 5. Affinity chromatography of keratan sulphate-protein cores on HA-Sepharose 2B. Proteoglycan monomer from normal nucleus pulposus (A), normal annulus fibrosus (B), degenerate nucleus pulposus (C) and degenerate annulus fibrosus (D) were digested with chondroitinase ABC and applied to an HA-Sepharose 2B affinity column. The column was washed with 60 ml of 0.5 M guanidinium chloride per 0.05 M sodium acetate (pH 5.8). At the point indicated by the arrow, the concentration was increased to 4 M guanidinium chloride. Column effluent was monitored by uronic acid analysis.

Fig. 6. Gel filtration of keratan sulphate protein cores on Sepharose 4B. Chondroitinase ABC-digested proteoglycan monomer from normal nucleus pulposus (A), normal annulus fibrosus (B), degenerate nucleus pulposus (C) and degenerate annulus fibrosus (D) were applied to an HA-Sepharose 2B affinity column. 0.5 M GuCl effluent (- - - - -) and 4 M GuCl effluent (———) were recovered, dialysed, lyophilised and chromatographed on Sepharose 4B (0.6 × 150 cm). Column effluents were analysed for uronic acid. The K_d values were: normal nucleus pulposus, 0.5 M GuCl 0.8, 4 M GuCl 0.56; normal annulus fibrosus, 0.5 M GuCl 077; 4 M GuCl 0.46; degenerate nucleus pulposus, 0.5 M GuCl 0.67; 4 M GuCl 0.37; degenerate annulus fibrosus, 0.5 M GuCl 0.71; 4 M GuCl 0.41. CuCl, guanidinium chloride.

Discussion

Age-related changes occurring in the intervertebral disc include a decrease in total polysaacharide content of the tissue, a fall in water content and an increase in collagen content. The average hydrodynamic size of the proteoglycan monomer decreases with maturation while the molecule becomes richer in keratan sulphate and poorer in chondroitin sulphate [40,41]. In articular carti-

lage the proportion of proteoglycans capable of interaction with hyaluronate does not change [42].

Differences in the chemical composition of normal and degenerate intervertebral disc were observed, particularly in the nucleus pulposus. Some of the changes are characteristic of aging tissue: there was a decrease in the water and uronic acid contents (Fig. 1A and Fig. 1D), notably in the nucleus and inner annulus of the degenerate disc, and an increased ratio of glucosamine to galactosamine (Table I). Collagen content increased slightly in the degenerate nucleus and decreased in the degenerate mid-annulus. The trend in collagen distribution across normal and degenerated disc was similar, increasing from the centre of the disc towards the periphery (Fig. 1C). However, the proteoglycan monomer displayed an apparent increase rather than a decrease in hydrodynamic size (Fig. 3). In addition a far greater proportion of proteoglycans of degenerate disc were capable of aggregation (Fig. 5).

Oegema et al. [37] have shown that newly-synthesized proteoglycans of human nucleus pulposus are of large molecular weight and are capable of aggregation; subsequent degradation of these molecules is likely to give rise to a variety of smaller and/or non-aggregating proteoglycans. Since the proteoglycans of degenerate disc are of greater average hydrodynamic size and include more molecules capable of aggregation, it follows that a larger proportion of these are newly synthesized. Normal (albeit younger) tissue, contained proteoglycans of smaller average size. As fewer of these were capable of aggregation it is likely that they have undergone a degree of degradation. It may be that synthesis in normal tissue is slower than in degenerate tissue since a greater proportion of proteoglycans in normal tissue are in a degraded state. The fact that aggregating keratan sulphate-protein cores are larger than non-aggregating cores (Fig. 6) suggests that degradation occurred to a definite extent in the hyaluronate binding region. The fact that the average chondroitin sulphate chain size was the same in all samples examined (Fig. 4) rules out any role chondroitin sulphate chains might play in the observed differences in hydrodynamic size of the intact proteoglycans.

The higher ratio of keratan sulphate to chondroitin sulphate in proteoglycans of degenerate tissue, however, was more likely an age-related phenomnon.

This study has not provided any explanation for the observed decrease in water content of degenerate intervertebral discs. Although the uronic acid concentration, which reflects charge density, is higher in normal than in degenerate discs, swelling pressure measurements show that the swelling potential of both normal and degenerate discs is not fully realised in vivo. It is suggested that other factors are operative in regulating the degree of hydration of intervertebral discs.

The mechanical efficiency of the disc depends on the integrity of the collagen framework; biochemical changes within the network could be responsible for degeneration and/or prolapse [43]. The variation in collagen content across normal and degenerate intervertebral discs was slight. The observation that swollen degenerate discs had a lower water content than swollen normal discs (Fig. 1B) speaks against any mechanical disruption or fracture of the collagen network as observed in osteoarthrosis [44]. It is more likely that chemical dif-

ferences in disc collagen are implicated in mechanical failure of the intervertebral disc [11,45]. These changes may include variations in the relative proportions and distributions of Type I and Type II collagens, the extent of hydroxylation or the degree of fibril cross-linking [45]. Proteoglycans may regulate the size and deposition of collagen fibrils [10]. The variable proteoglycans in normal and degenerate discs could alter the nature of the collagen network, assuming collagen turnover still occurs in this tissue. Increased susceptibility to enzyme degradation and the appearance of immature fibre types in electron microscopy suggests collagen synthesis in degenerate disc [29].

In a 'hydrostatic' model of the intervertebral joint, the nucleus pulposus is assumed to be gelatinous and a perfect hydrostatic medium, so that on application of a compressive load, the nuclear cavity develops an internal pressure which is converted into tensile stress distributed to the annulus fibres equally in all directions [46]. It has been shown that with disc degeneration, the water and uronic acid content, particularly in the centre of the disc, are reduced. The nucleus thus becomes more fibrotic and water and uronic acid concentration gradients, which were observed across the diameter of the normal disc, become less steep. The nucleus of the degenerate disc is thus likely to behave non-hydrostatically when subjected to load [46]. As physical differences between the nucleus and the annulus become reduced with degeneration, compressive stresses would be developed in both in the direction of the applied load and tractional in the radial direction. The failure of the annulus fibrosus would, under such conditions, be due to separation of the fibres themselves because the inter-fibre medium is likely to have less tensile strength than that of the fibres.

Acknowledgments

This work was supported by the South African Council for Scientific and Industrial Research and the South African Medical Research Council. We thank Ferelyth Douglas for help in preparing the manuscript.

References

1 Szirmai, J.A. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 3, (Balazs, E.A. ed.), pp. 1279—1308, Academic Press, London
2 Nachemson, A. (1976) in The Lumbar Spine and Back Pain, (Malcolm Jayson, ed.), pp. 355—365, Pitman Medical, London
3 Kellgren, J.H. (1977) Rheumatol. Rehab. 16, 3—12
4 Vernon-Roberts, B. and Pirie, C.J. (1977) Rheumatol. Rehab. 16, 13—21
5 Maroudas, A., Stockwell, R.A., Nachemson, A. and Urban, J. (1975) J. Anat. 120, 113—130
6 Eyring, E.J. (1969) Clin. Orthop. 67, 16—28
7 Jackson, D.S. (1978) J. Clin. Path., supplement (Royal College of Pathologists) 12, 44—48
8 Bailey, A.J. (1978) J. Clin. Path., supplement (Royal College of Pathologists) 12, 49—58
9 Ireland, J.T. (1978) J. Clin. Path., supplement (Royal College of Pathologists) 12, 59—66
10 Muir, I.H.M. (1979) in Adult Articular Cartilage, 2nd edition, (Freeman, M.A.R., ed.), pp. 145—214, Pitman Medical, London
11 Adams, P., Eyre, D.R. and Muir, H. (1977) Rheumatol. Rehab. 16, 22—29
12 Coventry, M.B. (1969) Clin. Orthop. Relat. Res. 67, 9—15
13 Harris, R.I. and Macnab, I. (1954) J. Bone Jt. Surg. 36, 304—322
14 Immelman, A.R., Sweet, M.B.E. and Thonar, E.J.-M.A. (1978) S. Afr. J. Med. Lab. Technol. 24, 35—36

15 Bitter, T. and Muir, H.M. (1962) Anal. Biochem. 4, 330—334
16 Grant, R.A. (1964) J. Clin. Path. 17, 685—686
17 Woessner, J.F. (1961) Arch. Biochem. Biophys. 93, 440—447
18 Jourdian, G.W., Dean. L. and Roseman, S. (1971) J. Biol. Chem. 246, 430—435
19 Urban, J.P.G. and Maroudas, A. (1979) Biochim. Biophys. Acta 586, 166—178
20 Venn, M. and Maroudas, A. (1977) Ann. Rheum. Dis 36, 121—129
21 Ghosh, P., Taylor, T.F.K., Braund, K.G. and Larson, L.H. (1976) Vet. Pathol. 13, 414—417
22 Scott, J.E. (1960) Methods Biochem. Anal. 8, 145—197
23 Oegema, T.R., Hascall, V.C. and Dziewiatkowski, D.D. (1975) J. Biol. Chem. 250, 6151—6159
24 Antonopoulos, C.A., Axelson, I., Heinegard, D. and Gardell, S. (1974) Biochim. Biophys. Acta 338, 108—119
25 Thonar, E.J.-M.A. and Sweet, M.B.E. (1979) Biochim. Biophys. Acta 584, 353—357
26 Sweet, M.B.E., Thonar, E.J.-M.A. and Marsh, J. (1979) Arch. Biochem. Biophys. 198, 439—448
27 Heinegard, D. and Hascall, V.C. (1974) Arch. Biochem. Biophys. 165, 427—441
28 Christner, J.E., Brown, M.L. and Dziewiatkowski, D.D. (1978) Anal. Biochem. 90, 22—32
29 Naylor, A. (1971) Orthop. Clin. North Am. 2,2, 343—358
30 Pearce, R.H. and Grimmer, B.J. (1976) Biochem. J. 157, 753—763
31 Stevens, R.L., Ewins, R.J.F., Revell, P.A. and Muir, H. (1979) Biochem. J. 179, 561—572
32 Hardingham, T.E. and Muir, H. (1972) Biochim. Biophys. Acta 279, 401—405
33 Hascall, V.C. and Heinegard, D. (1974) J. Biol. Chem. 249, 4232—4241
34 Preston, B.N. and Snowden, J.M. (1972) Biopolymer 11, 1627—1643
35 Maroudas, A. (1973) in Adult Articular Cartilage, (Freeman, M.A.R., ed.), pp. 131—170, Ptiman Medical, London
36 Glatz, C.E. and Massaro, T.A. (1976) Atherosclerosis 25, 165—173
37 Oegema, T.R., Bradford, D.S. and Cooper, K.M. (1979) J. Biol. Chem. 254, 10579—10581
38 Wasteson, A. (1971) J. Chromatography 59, 87—97
39 Bushell, G.R., Ghosh, P., Taylor, T.F.K. and Akeson, W.H. (1977) Clin. Orthop. Relat. Res. 129, 115—123
40 Lyons, J., Jones, E., Quinn, F.E. and Sprunt, D.H. (1966) J. Lab. Clin. Med. 68, 930—939
41 Adams, P. and Muir, H. (1976) Ann. Rheum. Dis. 35, 289—296
42 Inerot, S., Heinegard, D., Audell, L. and Olsson, S.-E. (1978) Biochem. J. 169, 143—156
43 Bailey, A.J., Herbert, C.M. and Jayson, M.I.V. (1976) in The Lumbar Spine and Back Pain, (Malcolm Jayson, ed.), pp. 327—340, Pitman Medical, London
44 Freeman, M.A.R. and Meachim. G. (1979) in Adult Articular Cartilage, 2nd edition (Freeman, M.A.R., ed.), pp. 487—543, Pitman Medical, London
45 Eyre, D.R. (1979) in International Review of Connective Tissue Research, Vol. 8, (Hall, D.A. and Jackson, D.S., eds.), pp. 227—291, Academic Press, London
46 Shah, J.S. (1976) in The Lumbar Spine and Back Pain, (Malcolm Jayson, ed.), pp. 271—292, Pitman Medical, London