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Structure of proteoglycans from different layers of human articular cartilage

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Full-depth plugs of adult human articular cartilage were cut into serial slices from the articular surface and analysed for their glycosaminoglycan content. The amount of chondroitin sulphate was highest in the mid-zone, whereas keratan sulphate increased progressively through the depth. Proteoglycans were isolated from each layer by extraction with 4M-guanidinium chloride followed by centrifugation in 0.4M-guanidinium chloride/CsCl at a starting density of 1.5g/ml. The efficiency with which proteoglycans were extracted depended on slice thickness, and extraction was complete only when cartilage from each zone was sectioned at 20μm or less. When thick sections (250μm) were extracted, hyaluronic acid was retained in the tissue. Most of the proteoglycans, extracted from each layer under optimum conditions, could interact with hyaluronic acid to form aggregates, although the extent of aggregation was less in the deeper layers. Two pools of proteoglycan were identified in all layers by gel chromatography ($K_{av.}$ 0.33 and 0.58). The smaller of these was rich in keratan sulphate and protein, and gradually increased in proportion through the cartilage depth. Chondroitin sulphate chain size was constant in all regions. The changes in composition and structure observed were consistent with the current model for hyaline-cartilage proteoglycans and were similar to those observed with increasing age in human articular cartilage.

Proteoglycans are major constituents of the intercellular matrix of cartilage and are largely responsible for the high elasticity and resilience of the tissue (Kempson *et al.*, 1971; Scott, 1975; Kempson, 1975, 1979). The proteoglycan molecule consists of a central protein core to which a large number of negatively charged glycosaminoglycan side chains are covalently attached.

Stockwell & Scott (1967) used a combination of chemical and histochemical methods to show that the glycosaminoglycans in human articular cartilage varied through the tissue depth. Similar studies since then have also demonstrated that the glycosaminoglycan content is highest in the mid-zones and that there is a differential distribution of chondroitin sulphate and keratan sulphate (Maroudas *et al.*, 1969, 1973; Lemperg *et al.*, 1972; Maroudas, 1979). More-complete information on the composition of adult human cartilage has since been obtained by relating the results of chemical analysis performed on the tissue to the results obtained for extracted proteoglycans (Maroudas *et al.*, 1980; Bayliss & Venn, 1980). During these preliminary investigations it became clear that the extractability of proteoglycans appeared to vary with distance from the articular surface and that this may have been related to the changes with depth in tissue composition and structure. There were also indications that the proportion and composition of extracted components depended on slice thickness.

The present work (1) studies the changes in the extractability of proteoglycans as a function of depth and slice thickness, (2) studies the structure and composition of the proteoglycans extracted from the different layers, and (3) relates the differences in extractability with depth and slice thickness to the size and structure of the proteoglycans present.

Materials and methods

Chemicals

All reagents were analytical grade and were supplied by BDH Chemicals, Poole, Dorset, U.K. *Streptomyces* hyaluronidase was obtained from Calbiochem–Behring Corp., La Jolla, CA, U.S.A.

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Tissue preparation

Cartilage was obtained from human femoral heads either at post-mortem or at operation for subcapital fracture of the neck of femur, and was stored frozen at −20°C until use. In order to determine biochemical variations with depth, full-depth cartilage plugs were cut with a cork-borer and excised from the subchondral bone, and 250 μm-thick slices were cut parallel to the cartilage surface with a freezing microtome. For the studies on proteoglycan structure full-thickness plugs of cartilage were removed from the pressure-bearing area of five femoral heads of approximately the same age (70–80 years). The plugs were sectioned at 250 μm as described above, and slices from corresponding layers were pooled. This procedure resulted in five or six layers for each experiment.

Extraction and purification of proteoglycans

The cartilage slices were extracted by one of the following methods.

Method 1. Cartilage slices (250 μm thick) were extracted directly with 4 M-guanidinium chloride in 0.1 M-sodium phosphate buffer, pH 7.0 (10 ml/0.5 g wet wt.), for 24 h at 4°C.

Method 2. Cartilage slices (250 μm thick) were diced with a scalpel on a moist Petri dish and then frozen in liquid N_2, and 250 mg portions were powdered in the micro-vials of a Spex Freezer Mill (Spex Industries, Metuchen, NJ, U.S.A.) for 5 s at the highest frequency (Bayliss & Ali, 1978*b*). The powder was then extracted as described above.

Method 3. Cartilage slices (250 μm thick) were diced with a scalpel and frozen in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7.0, in plastic rings (Ali, 1967; Evans *et al.*, 1967). The resulting disc of buffer containing the cartilage was then sectioned at 20 μm on a freezing cryostat, and the sections were collected directly into sterile bottles, and extracted with 4 M-guanidinium chloride/0.1 M-phosphate buffer, pH 7.0.

Method 4. Cartilage slices (250 μM thick) were frozen in buffer as described for method 3, and sectioned at 2 μm before extraction with 4 M-guanidinium chloride / 0.1 M-phosphate buffer, pH 7.0. After extraction, the cartilage residues were allowed to settle (method 1), centrifuged (method 2), or removed by filtration on glass-wool (methods 3 and 4). The residues were washed with 5 ml of cold 4 M-guanidinium chloride/0.1 M-phosphate buffer, pH 7.0, and the washing and extracts were combined. The extracts were then dialysed against 9 vol. of 0.1 M-sodium phosphate buffer, pH 7.0, at 4°C.

The density of each extract was adjusted to 1.5 g/ml with solid CsCl, and the extracts were centrifuged at 95000 $g_{av.}$ for 48 h at 10°C in an 8 × 25 ml angle rotor in an MSE Superspeed 65 centrifuge. Each gradient was fractionated into a high-density fraction A_1 (5 ml) and a low-density fraction A_2 (13 ml) by upward displacement (Bayliss & Ali, 1978*b*). After dialysis against 0.1 M-sodium phosphate buffer, pH 7.0, to remove CsCl, the fractions were stored at −20°C until required. Cartilage residues remaining after the extraction were digested with papain (McDevitt *et al.*, 1974), and the resulting glycosaminoglycans were precipitated by the addition of 3 vol. of ethanolic 0.5 M-potassium acetate. The percentage fraction uronic acid extracted was calculated from the sum of uronic acid in the extracts and residues.

Gel chromatography

Proteoglycan samples (1 ml) were applied to a column (153 cm × 0.80 cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl at 2.5 ml/h at 4°C. Fractions (1 ml) were collected, and their uronic acid, hexose and protein contents were determined. Proteoglycan aggregates and glucuronolactone were used as markers of the void volume and total volume of the column respectively. The proportion of aggregates and monomers in the A_1 fractions was estimated by cutting and weighing a tracing of the uronic acid elution profile (Hardingham & Muir, 1974). Samples of purified proteoglycan fraction A_1 prepared by method 1 were mixed overnight with hyaluronic acid (2% on the basis of uronic acid content), and the extent of interaction was determined by gel chromatography on Sepharose 2B as described above.

The size distribution of the proteoglycan subunits in an A_1 preparation was determined on columns of Sepharose 2B after reduction and alkylation by the method of Heinegård (1977). A sample of fraction A_1 (400 μg of uronic acid) in 4 M-guanidinium chloride/0.1 M-phosphate buffer, pH 7.0, was reduced with 5 mM-dithiothreitol for 5 h at 37°C and alkylated with 15 mM-iodoacetate for 17 h at 22°C.

The size distribution of chondroitin sulphate chains in fraction A_1 and the cartilage residues was determined on a column (0.55 cm × 110 cm) of Sephacryl S-300 after digestion with papain.

Polyacrylamide/agarose-gel electrophoresis

Large-pore polyacrylamide/agarose gels were prepared as described by McDevitt & Muir (1971). The gels were cut to 7 cm before electrophoresis. Samples of fraction A_1 (25 μl, containing 2–3 μg of uronic acid) were disaggregated in 4 M-urea/0.1 M-phosphate buffer, pH 7.0, at 22°C for 48 h, and then layered on the gels in 20% (w/v) sucrose containing 0.05% Bromophenol Blue. Electrophoresis was performed in 10 mM-Tris/acetate buffer, pH 6.8, containing 4 M-urea, 0.25 mM-Na_2SO_4 and 1 mM-EDTA at 4 mA/gel until the Bromophenol Blue had

migrated 3 cm. The gels were stained with Toluidine Blue and destained with 3% (v/v) acetic acid and water.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Polyacrylamide running gels (7.5%) with a 3.3% stacking gel were prepared by the method of Weber & Osborn (1969). Samples of A_1 fractions from each layer containing 100 μg of protein in 50 μl of 1% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.0, were heated at 100°C for 3 min and then electrophoresed for 7 h at a current of 8 mA/gel. The gels were stained with Coomassie Blue R-250 and destained in 7.5% (v/v) acetic acid at 37°C.

Enzyme treatment

To determine the content of hyaluronic acid in proteoglycan preparations, samples of the A_1 fractions were precipitated with ethanolic 0.5 M-potassium acetate, resuspended in 50 mM-sodium acetate buffer, pH 5.0, and incubated with *Streptomyces* hyaluronidase [5 TRU (turbidity-reducing units)/ml] for 24 h at 37°C. Undigested products were precipitated as before and used for recombination experiments on proteoglycans extracted by method 1.

Determination of dry weight and glycosaminoglycan distribution

Adjacent plugs of cartilage were used to compare extracted and non-extracted samples. Slices for extraction (approx. 20 mg wet wt.) were placed in 2 ml of 4 M-guanidinium chloride/0.1 M-phosphate buffer, pH 7.0, and shaken for 24 h at 4°C, and they were then washed several times with 0.15 M-NaCl and their wet weight and glycosaminoglycan content determined. Dry weight was measured after drying of samples to constant weight at 67°C. Slices were digested with papain (Hjertquist & Lemperg, 1967) and analysed for hexosamine and uronic acid.

Hexosamine was determined by the Elson & Morgan (1933) reaction after hydrolysis in 6 M-HCl for 4 h at 100°C. Uronic acid was measured by the method of Bitter & Muir (1962). In order to convert the chemical results into weights of keratan sulphate and chondroitin sulphate, for chondroitin sulphate the uronic acid results were multiplied by the factor 513 (the molecular weight of the disaccharide), and for keratan sulphate the difference between hexosamine and uronic acid values (mmol/g wet wt. of tissue) was multiplied by the factor 464 (Venn & Maroudas, 1977).

Chemical methods

The chemical analyses of purified proteoglycans and column effluents were performed on a Technicon AAII AutoAnalyzer by using modifications (M. T. Bayliss, unpublished work) of the following methods: uronic acid (Bitter & Muir, 1962); protein (Lowry *et al.*, 1951); galactosamine and glucosamine (Blumenkrantz & Asboe-Hansen, 1976; Wagner, 1979); hexose (Trevelyan & Harrison, 1952). Samples for hexosamine analysis were hydrolysed for 3 h at 95°C in 8 M-HCl before rotary evaporation.

Results

Extractability of chondroitin sulphate and keratan sulphate from 250 μm-thick slices

Fig. 1 shows the chondroitin sulphate and keratan sulphate contents of successive 250 μm-thick slices both before and after extraction. Before extraction the amount of chondroitin sulphate first increases with distance from the articular surface, then declines again in the deep zone, whereas keratan sulphate shows a steady increase with depth. This results in a higher overall glycosaminoglycan con-

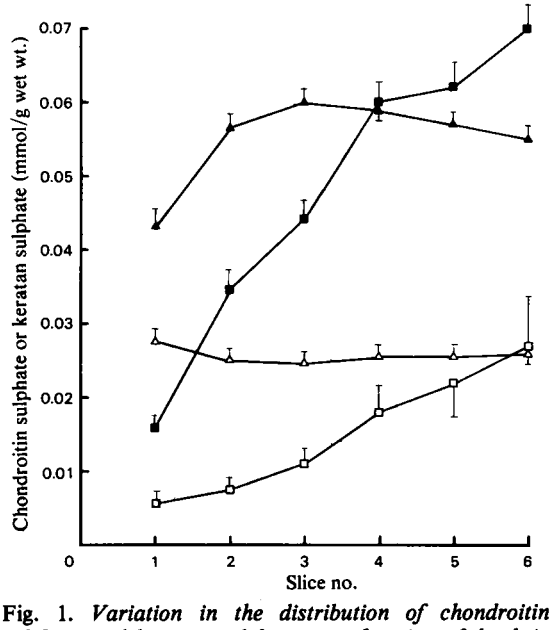

Fig. 1. *Variation in the distribution of chondroitin sulphate and keratan sulphate as a function of depth in articular cartilage, before and after extraction*

Individual samples of full-depth cartilage were sectioned into 250 μm-thick slices from the articular surface (slice 1) and analysed for uronic acid (▲) and hexosamine (■). Slices from adjacent samples were extracted for 24 h with 4 M-guanidinium chloride before determination of their uronic acid (△) and hexosamine (□) contents. For experimental details see the text. The bars indicate S.E.M. for results from five femoral heads.

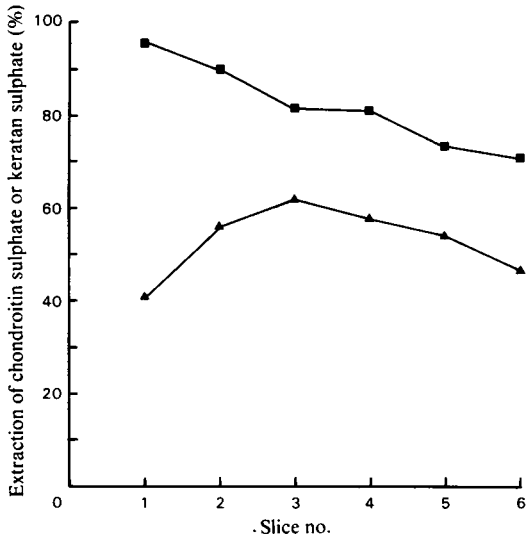

Fig. 2. *Extractability of chondroitin sulphate and keratan sulphate as a function of depth in articular cartilage*
The percentage extraction of chondroitin sulphate (▲) and keratan sulphate (■) from the slices described in Fig. 1 was calculated as the difference in values before and after extraction with 4 M-guanidinium chloride. For experimental details see the text.

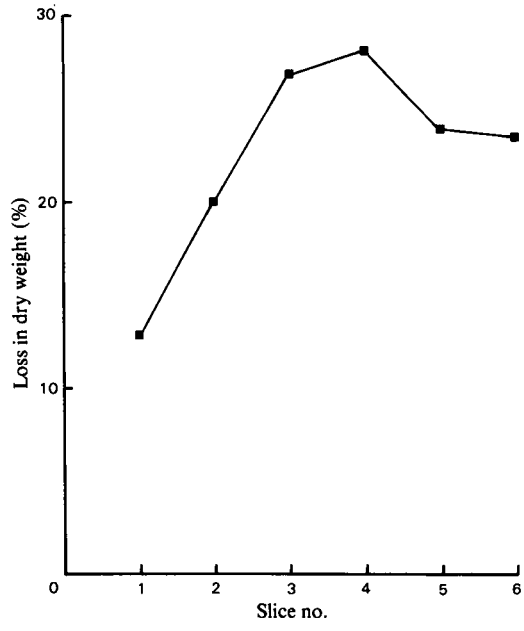

Fig. 3. *Loss in dry weight of cartilage slices as a function of depth, after extraction with 4 M-guanidinium chloride*
The percentage decrease in dry weight of the cartilage samples described in Fig. 1 was calculated after extraction of the slices with 4 M-guanidinium chloride for 24 h. For experimental details see the text.

tent in the middle and deep regions, which is consistent with previous data on adult human cartilage (Stockwell & Scott, 1967; Maroudas *et al.*, 1969, 1980; Maroudas, 1979). In the extracted slices, on the other hand, chondroitin sulphate is practically constant throughout the cartilage thickness, whereas the increase in keratan sulphate is much less pronounced than in the total tissue. It is clear that there is a preferential extraction of keratan sulphate throughout the cartilage thickness, and this effect is particularly pronounced in the surface zone (Fig. 2). There is an overall increase in the percentage extraction from the surface to the middle zone for chondroitin sulphate, but an overall decrease for keratan sulphate.

Loss in dry weight

The loss in dry weight, plotted in Fig. 3 versus distance from the articular surface, shows a maximum (28%) in the middle zone, where the absolute amounts of both chondroitin sulphate and keratan sulphate extracted are also at a maximum. Since the total glycosaminoglycan content in the middle zone of the femoral head is about 22% of the dry weight (Maroudas *et al.*, 1980), and since only 60–70% of the glycosaminoglycans is extracted, a considerable fraction of the extracted material must consist of protein.

Effect of cartilage pretreatment on extraction of proteoglycans

Extraction of the pooled 250 μm-thick slices used for the preparation of proteoglycans gave essentially the same results as found for the single slices described above (Fig. 4). The extraction yield was increased in all slices, most noticeably in the surface and deep regions, by powdering them in liquid N_2 (method 2) before extraction, corroborating previous results on full-thickness cartilage (Bayliss & Ali, 1978*b*). Similarly, treatment of the slices by sectioning them at 20 μm (method 3) or at 2 μm (method 4) increased the extraction yield even further, so that 80–85% of the uronic acid was removed from each layer.

Analysis of A_1 fractions

The glycosaminoglycan and protein contents of the A_1 fraction prepared by each method were determined and are shown in Table 1. Fraction A_1 accounted for approximately the same proportion of uronic acid on each gradient, irrespective of the layer or method of cartilage preparation. The protein distribution, however, did show a zonal variation, and the amount present in fraction A_1 from the deep

Table 1, *Analysis of proteoglycans (A_1 fractions) isolated from different layers of human articular cartilage*
For experimental details see the text. Galactosamine/glucosamine ratios given in parentheses refer to cartilage residues. N.D., Not determined.

	Slice no. from articular surface (250 μm)	Uronic acid (% of total gradient)	Protein (% of total gradient)	Protein (Lowry)/ uronic acid weight ratio	Galactosamine/ glucosamine molar ratio	Uronic acid excluded from Sepharose 2B column (%)
Method 1, Expt. (iii), 1st extract	1	88.4	54.1	1.57	1.55	14
	2	92.0	64.2	1.62	1.33	19
	3	92.8	66.3	1.86	1.15	17
	4	90.7	65.2	2.04	1.05	26
	5	91.6	66.2	2.22	0.86	30
Method 1, Expt. (iii), 2nd extract	1	92.2	N.D.	2.33	1.79 (1.76)	N.D.
	2	91.8	N.D.	2.39	1.53 (1.54)	N.D.
	3	91.6	N.D.	2.60	1.31 (1.47)	N.D.
	4	89.5	N.D.	2.51	1.14 (1.14)	N.D.
	5	92.5	N.D.	3.47	1.60 (1.28)	N.D.
Method 2	1	83.4	53.4	3.86	1.30	70
	2	86.4	59.5	3.66	1.02	68
	3	89.5	63.2	4.00	0.75	68
	4	91.5	61.4	4.26	0.72	65
	5	88.3	64.0	4.32	0.68	44
Method 3	1	85.1	50.6	3.37	1.60	66
	2	86.9	52.9	3.33	1.17	63
	3	88.3	52.8	3.69	0.94	56
	4	91.4	60.2	4.36	0.82	53
	5	88.2	61.8	4.43	0.71	55
Method 4	1	93.8	60.7	2.05	1.44 (2.65)	53
	2	95.3	61.7	2.08	1.04 (1.32)	52
	3	90.1	64.2	2.19	0.89 (1.07)	52
	4	94.8	64.5	2.51	0.77 (1.04)	51
	5	96.6	67.1	2.94	0.74 (0.89)	43

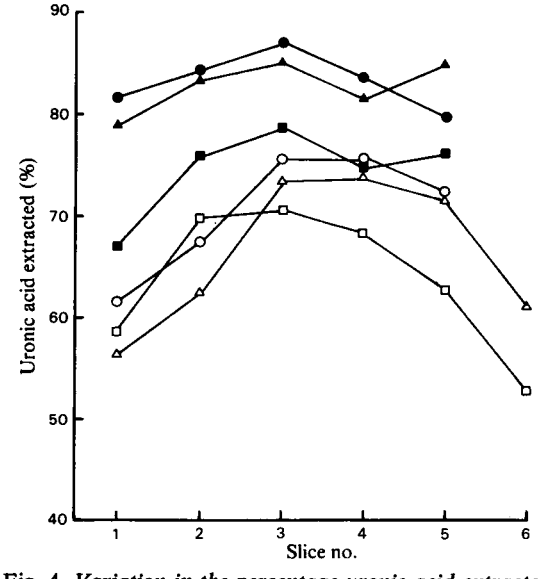

Fig. 4. *Variation in the percentage uronic acid extracted as a function of depth in normal articular cartilage*
Slices of cartilage (250 μm) were pretreated as described in the Materials and methods section before extraction with 4 M-guanidinium chloride, pH 7.0 (slice 1, articular surface). For experimental details see the text. □, Method 1, Expt. (i); △, method 1, Expt. (ii); ○, method 1, Expt. (iii); ■, method 2; ▲, method 3; ●, method 4.

layers was generally 10% higher than at the surface. Similarly, the protein/uronic acid weight ratios increased through the cartilage depth, and this finding was again independent of the extraction method and indicative of a higher protein content for the proteoglycans from the deeper regions. Concomitant with this change was a decrease in the galactosamine/glucosamine molar ratios of the A_1 fractions, indicating an increase in keratan sulphate content relative to chondroitin sulphate, consistent with the analyses of non-extracted slices. The cartilage residues after extraction contained relatively more galactosaminoglycans than did the purified proteoglycans, which was contrary to the results obtained by Inerot *et al.* (1978) and Franzén *et al.* (1981).

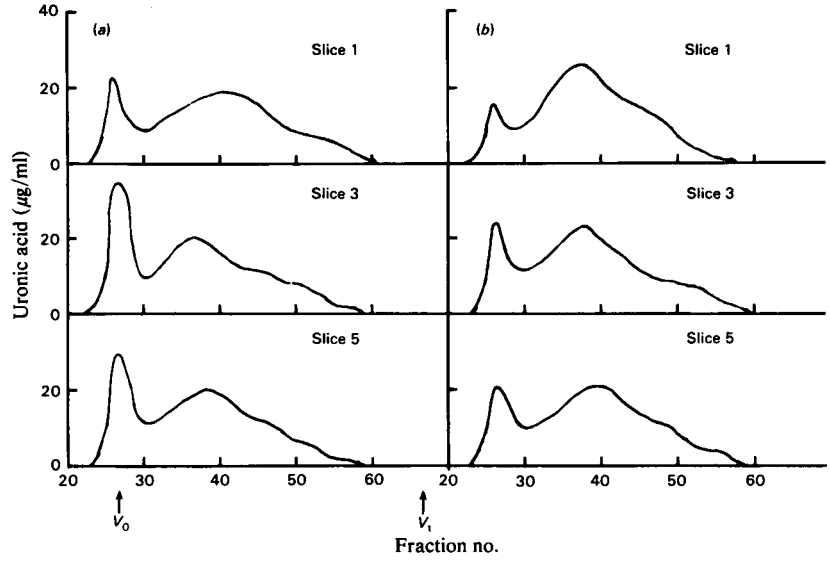

Fig. 5. *Gel chromatography on Sepharose 2B of A_1 fractions from articular cartilage prepared by method 1, Expts. (i) and (ii)*

Samples of the A_1 fractions from (*a*) Expt. (i) and (*b*) Expt. (ii) were applied to a column (153 cm × 0.8 cm) of Sepharose 2B and eluted with 0.1 M-NaCl/0.1 M-phosphate buffer, pH 7.0, as described in the text. The uronic acid (——) content of each 1 ml fraction was determined. For experimental details see the text. V_0 and V_t mark the void volume and total volume of the column respectively. Slice 1 and slice 6 are the surface and deepest layers respectively. The profiles for slices 2, 4 and 6 closely resembled those of slices 3 and 5.

Gel chromatography

Although the zonal variations in composition were similar for the different methods of tissue preparation, the macromolecular organization of the proteoglycans was dependent to a large extent on the pre-extraction conditions.

Extraction of 250 μm-thick slices (method 1)

Fig. 5 shows the chromatographic profiles for the preparations from Expts. (i) and (ii), obtained by method 1. Only a small proportion of the proteoglycans extracted by this method was eluted in the void volume of the column (Table 1), demonstrating that only a few of the proteoglycan molecules were in an aggregate form. The surface layer invariably had the lowest aggregate content, but even in the deeper layers the proportion rarely exceeded 30%. The uronic acid profiles of the proteoglycans retarded on the column showed them to be polydisperse, and two populations of different hydrodynamic size were identifiable in each layer of cartilage. The smaller of these had a higher protein content than had the larger species.

A more detailed chromatographic analysis of the proteoglycans extracted by this method was performed [Expt. (iii), method 1], the results of which are shown in Fig. 6(*a*). Of the two proteoglycan pools, the lower-molecular-weight one had a high hexose content, probably due to keratan sulphate. Fig. 6(*b*) shows that under aggregating conditions most of the uronic acid was eluted in the column void volume, confirming that most of the proteoglycans extracted by method 1 had a functional hyaluronic acid-binding region and that no degradation of this part of the molecule had occurred during extraction.

The potential of the proteoglycans to aggregate, and previous studies on full-thickness human cartilage in which a high aggregate content was observed (Bayliss & Ali, 1978*b*), suggested that by using method 1 preferential extraction of proteoglycans had taken place and that most of the tissue hyaluronic acid was retained in the 250 μm-thick slices. In order to investigate this hypothesis further, the 250 μm-thick slices remaining after extraction by method 1 [Expt. (iii)] were sectioned at 20 μm and extracted for a second time with 4 M-guanidinium chloride. The uronic acid profiles for the A_1 fractions on Sepharose 2B are shown in Fig. 7(*a*), and indicate elution as a very polydisperse retarded peak. The hexose and protein distributions (not shown) were the same as that of uronic acid. Addition of hyaluronic acid to the A_1 fractions isolated from the second extracts had no effect on the chromatographic profiles (results not shown). If, however, the A_1 fractions of corresponding layers from the first

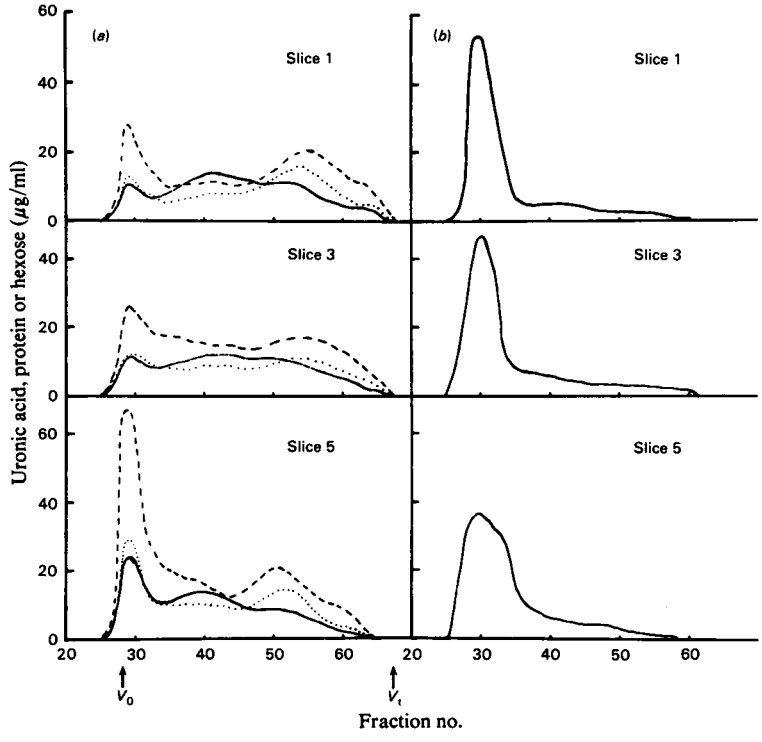

Fig. 6. *Gel chromatography on Sepharose 2B of A_1 fractions from articular cartilage prepared by method 1, Expt. (iii) (first extract)*

(*a*) The A_1 fractions prepared from the first extract of Expt. (iii) (method 1) were chromatographed on a column (153 cm × 0.8 cm) of Sepharose 2B as described in the text, and 1 ml fractions were analysed for uronic acid (——), protein (– – – –) and hexose (·····). (*b*) Samples of the A_1 fractions described above for (*a*) were incubated with hyaluronic acid before chromatography on Sepharose 2B. Fractions (1 ml) were assayed for uronic acid (——). For experimental details see the text.

and second extracts were combined in the correct proportion (based on the percentage of the total extractable uronic acid that they represented), then most of the proteoglycans were excluded from the column as aggregates (Fig. 7*b*). Further evidence that the aggregating component in the second extract was hyaluronic acid was obtained by digesting a sample of these fractions with *Streptomyces* hyaluronidase, whereon subsequent recombination with the first extracts did not result in aggregation. The control sample incubated with heat-inactivated enzyme did form aggregates, as expected. Furthermore, incubation of the first extracts with hyaluronidase did not alter their ability to aggregate, ruling out the possibility of partial degradation of the proteoglycans by contaminating proteinases in the enzyme preparation.

Subsequent methods aimed at providing a more complete extraction of the tissue slices.

Extraction of powdered slices (method 2) or 20 μm-thick sections (method 3)

Both of these methods of tissue preparation gave essentially the same results. Unlike those isolated by method 1, the proteoglycans in fraction A_1 from each layer were largely aggregated and therefore excluded from Sepharose 2B columns (Figs. 8*a* and 8*b*). Furthermore, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the link proteins that are present in the aggregate preparation from full-thickness cartilage (Bayliss & Ali, 1978*b*) were also present in the A_1 fractions from each layer (Fig. 9). Additional features common to the preparations obtained by methods 2 and 3 were the relatively lower content of aggregates in the deeper layers and the increasing protein content of the proteoglycans through the cartilage depth (Table 1).

In order to study the size distribution of proteoglycans comprising the aggregates, a sample of

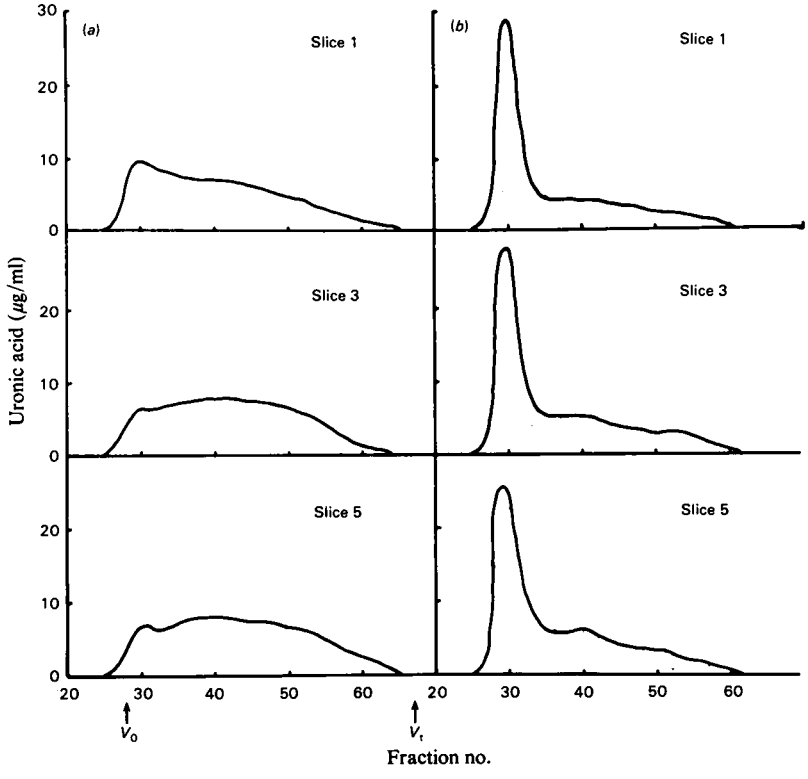

Fig. 7. *Gel chromatography on Sepharose 2B of A_1 fractions from articular cartilage prepared by method 1, Expt. (iii) (second extract)*

(*a*) Samples of A_1 fractions prepared from the second extract of Expt. (iii) (method 1) were chromatographed on a column (153 cm × 0.8 cm) of Sepharose 2B, and 1 ml fractions were analysed for uronic acid (——). (*b*) The A_1 fractions purified from the first and second extracts of corresponding layers of cartilage, described for Expt. (iii), were recombined (see the text) before gel chromatography on Sepharose 2B. Fractions (1 ml) were analysed for uronic acid (——). For experimental details see the text.

fraction A_1 from each layer was reduced and alkylated before chromatography on Sepharose 2B (Figs. 10*a* and 10*b*). Although the distribution was polydisperse, the two proteoglycan species described above for method 1 were again identified. In addition, the uronic acid and protein elution profiles further suggested that there was a small but significant increase in the lower-molecular-weight protein-rich proteoglycan species in the deeper layers.

Extraction of 2 μm-thick sections (method 4)

Although powdering and sectioning (20 μm) of cartilage increased the extraction efficiency considerably, there was still some 15–20% uronic acid remaining in the residue. To try and improve the yield still further, 250 μm-thick slices were sectioned at 2 μm. The elution profiles of the A_1 fraction on Sepharose 2B were very similar to those of the preparations obtained by methods 2 and 3, i.e. most of the proteoglycans were aggregated (Fig. 11*a*) and the proportion of aggregates tended to decrease through the cartilage depth. The changes in composition of these fractions, described in Table 1, were also reflected in the protein (results not shown) and hexose chromatographic elution profiles. The size distribution of reduced and alkylated proteoglycan was determined as before and indicated the presence of a large species and a small species, with $K_{av.}$ 0.33 and 0.58 respectively. There was a proportional increase in the smaller of the two species through the cartilage depth (Fig. 11 and Table 2). As described for the proteoglycans extracted by method 1, the smaller of the proteoglycan species had a higher content of hexose (keratan sulphate) and of protein than the larger species. To determine whether the changing size of the proteoglycans was partly the result of a change in chondroitin sulphate chain size,

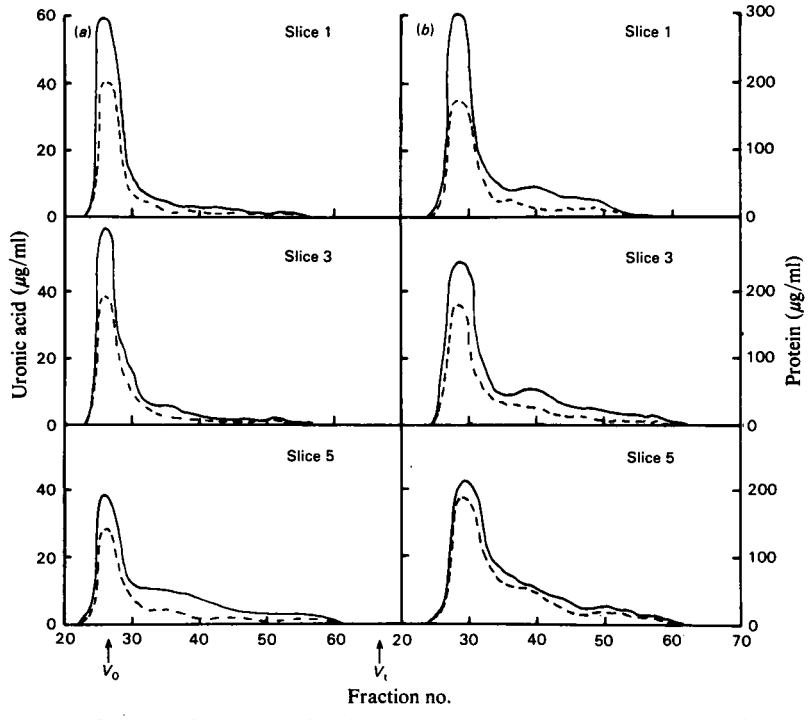

Fig. 8. *Gel chromatography on Sepharose 2B of A_1 fractions from articular cartilage prepared by methods 2 and 3*
Samples of A_1 fractions purified from each layer of cartilage by (*a*) method 2 and (*b*) method 3 were chromatographed on a column (153 cm × 0.8 cm) of Sepharose 2B. Fractions (1 ml) were analysed for uronic acid (——) and protein (– – – –). For experimental details see the text.

papain digests of the A_1 fractions were eluted on a column of Sephacryl S-300. The chromatograms show that the size distribution was the same in all zones.

Residual glycosaminoglycans

Sectioning cartilage slices at 2 μm did increase the extraction yield from the surface layer by a small amount, but there was very little change in other zones. Thus 15–20% of the uronic acid still remained in the residue. Analysis of the papain-digested residues showed that they had a higher galactosamine/glucosamine molar ratio than did the corresponding A_1 fractions, indicating a higher content of chondroitin sulphate. The size distribution of these chondroitin sulphate chains on Sephacryl S-300 was the same in different layers, but they were slightly larger (two column fractions) than those isolated from the extracted proteoglycans.

Gel electrophoresis

Electrophoresis in 4 M-urea of A_1 fractions isolated by method 4 enabled all the proteoglycans comprising the aggregate to be analysed. In each layer there were three bands, whose migration rates were constant and identical with those from full-thickness cartilage, but it was not possible to determine whether their proportions changed (Fig. 12). A slower-migrating species was also observed, which was very diffuse and whose migration rate increased in the middle and deep layers. This band was not present in preparations of proteoglycans purified by dissociative density-gradient centrifugation, and might represent the hyaluronic acid in the aggregate or alternatively might reflect an interaction of hyaluronic acid with the proteoglycans. The relatively high content of hyaluronic acid in adult human cartilage (Bayliss & Venn, 1980; Thonar *et al.*, 1978) would make both equally possible.

Discussion

It is clear from Fig. 1 that the percentage of keratan sulphate extracted from 250 μm-thick slices is much greater at all levels than that of chondroitin sulphate. This implies that the keratan sulphate-rich proteoglycans are the more readily extractable.

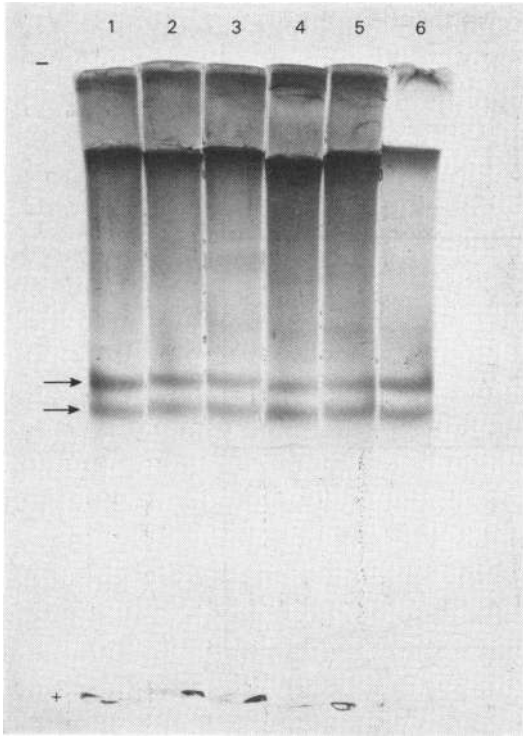

Fig. 9. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of A_1 fractions from articular cartilage prepared by method 3*

Experimental details are given in the Materials and methods section. Gels 1–5 correspond to the A_1 fractions prepared from slices 1–5 by method 3, and gel 6 is the A_1 fraction prepared from full-thickness cartilage. Gels were stained with Coomassie Blue R-250. The link proteins are arrowed.

Moreover, because the decrease in dry weight on extraction considerably exceeds the amount of glycosaminoglycan extracted, this indicates that the extracted proteoglycans are rich in protein. Since a very small fraction of collagen is lost on extraction, and since non-collagenous non-proteoglycan proteins constitute only a minor component of the tissue (Maroudas *et al.*, 1980), it is reasonable to assume that the decrease in dry weight is mainly due to proteoglycans. This in turn might account for some of the discrepancy in dry-weight measurements described by Muir *et al.* (1970). In some of their specimens as much as 50% of the dry weight of the deeper layers was not accounted for by collagen and glycosaminoglycans.

The studies on purified extracts show that the aggregating proteoglycans present in adult human articular cartilage consist of at least two species, which differ considerably in hydrodynamic size and composition, in marked contrast with those from other sources of hyaline cartilage, which are usually distributed as a single polydisperse peak when examined by gel chromatography. The current model for the structure of proteoglycans in hyaline cartilage attributes the variations in size and composition of the aggregating molecules primarily to changes in the length of the protein core and its substitution with glycosaminoglycan chains (Hascall & Sajdera, 1970; Tsiganos *et al.*, 1971; Heinegård & Hascall, 1974; Hardingham *et al.*, 1976; Rosenberg *et al.*, 1976; Heinegård, 1977). Consequently, as the size of the proteoglycan decreases, so the hyaluronic acid-binding region and keratan sulphate-rich region constitute a larger proportion of the molecule, and the relative contents of protein and

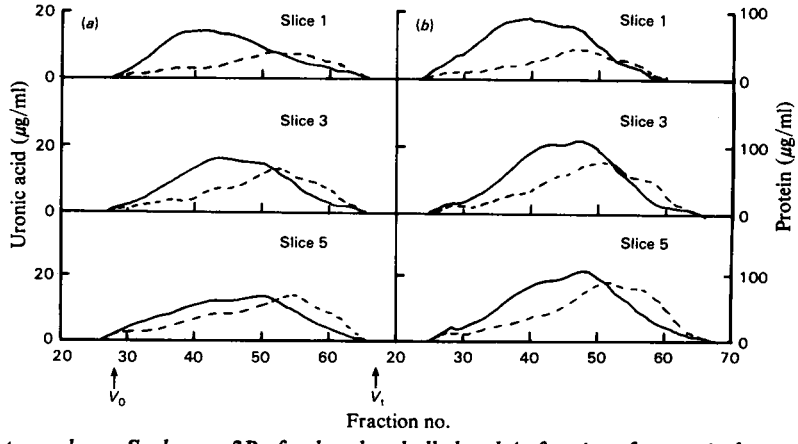

Fig. 10. *Gel chromatography on Sepharose 2B of reduced and alkylated A_1 fractions from articular cartilage prepared by methods 2 and 3*

The A_1 fractions purified by (*a*) method 2 and (*b*) method 3 were reduced and alkylated as described in the text and eluted from a column (153 cm × 0.8 cm) of Sepharose 2B. Fractions (1 ml) were analysed for uronic acid (——) and protein (– – – –). For experimental details see the text.

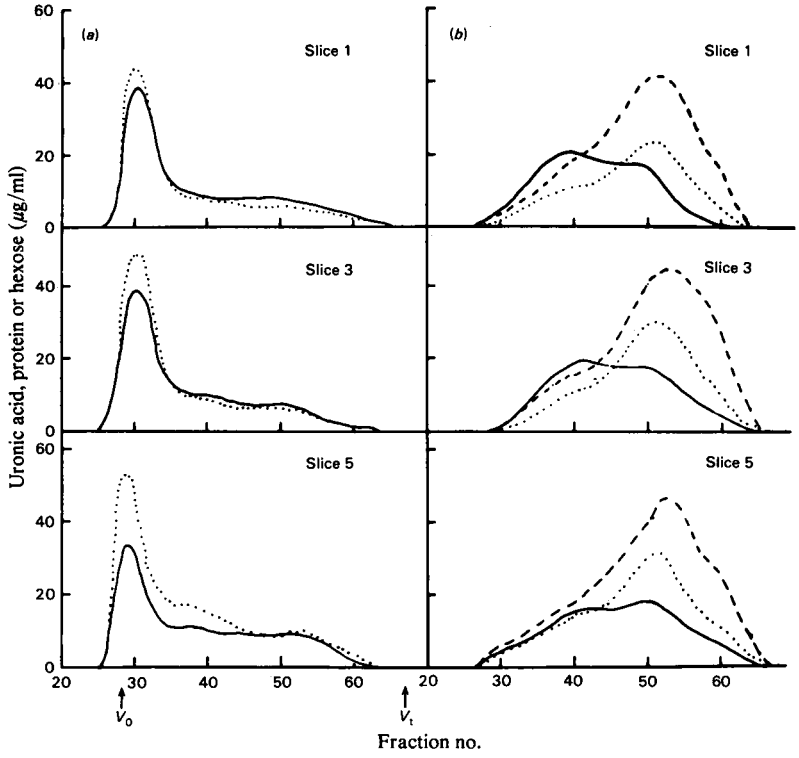

Fig. 11. *Gel chromatography on Sepharose 2B of A_1 fractions from articular cartilage prepared by method 4*
(*a*) A_1 fractions from each layer of cartilage, purified by method 4, were chromatographed on a column (153 cm × 0.8 cm) of Sepharose 2B. Fractions (1 ml) were analysed for uronic acid (——) and hexose (·····). (*b*) The A_1 fractions prepared by method 4 were reduced and alkylated and chromatographed as indicated above for (*a*). Fractions (1 ml) were analysed for uronic acid (——), protein (– – – –) and hexose (·····). For experimental details see the text.

Table 2. *Proportion of proteoglycan pools I and II in the chromatograms of reduced and alkylated A_1 fractions prepared by method 4*
For experimental details see the text. Values were determined by cutting and weighing a tracing of the chromatograms shown in Fig. 11(*b*); pool I is fractions 26–46, and pool II is fractions 47–65.

Slice no. from articular surface (250 μm)	Uronic acid (% of total chromatographed)	
	Pool I ($K_{av.} = 0.33$)	Pool II ($K_{av.} = 0.58$)
1	62	38
2	59	41
3	54	46
4	50	50
5	46	54

keratan sulphate increase. Thus the increasing protein and keratan sulphate contents of the A_1 fractions through the cartilage depth suggested a decrease in proteoglycan size, and a quantitative analysis of the uronic acid profiles, together with the protein and hexose profiles, confirmed that there was a marked increase in lower-molecular-weight proteoglycans in the deep layers. Essentially the same findings were made for bovine hip cartilage by Franzén *et al.* (1981), but, because of the thinner slices used in their study, they were also able to show that the upper 250 μm layer contained a much smaller range of proteoglycan sizes than did the remainder of the cartilage. The present results and those obtained by Franzén *et al.* (1981) showed identical chondroitin sulphate chain size through the depth and decreased aggregate content in the deep zone (possibly the result of cartilage matrix remodelling in the area close to the subchondral bone).

The proteoglycans in the A_1 fractions from each layer of cartilage also exhibited considerable heterogeneity on composite-gel electrophoresis, and confirmed previous studies on human cartilage (Bayliss & Ali, 1978*b*; Bouvet *et al.*, 1978; Peyron *et al.*, 1978; Roughley *et al.*, 1981). Although the results

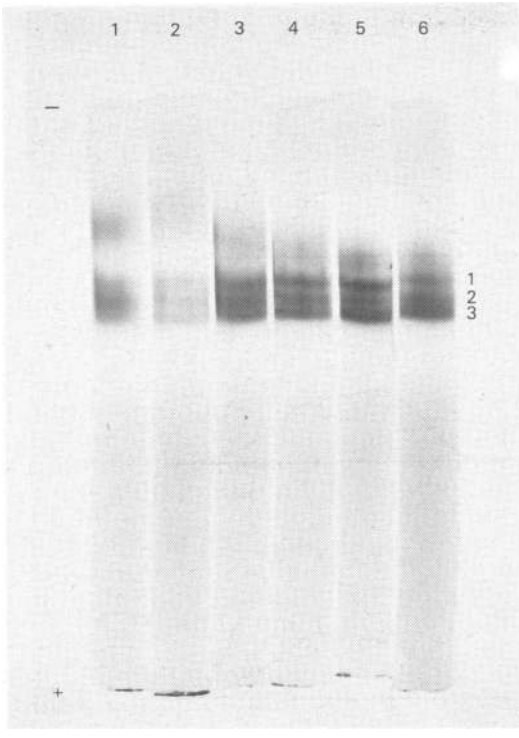

Fig. 12. *Agarose-(0.6%)/polyacrylamide-(1.2%)-gel electrophoresis of A_1 fractions from articular cartilage prepared by method 4*

Experimental details are given in the Materials and methods section. Gels 1–5 correspond to the A_1 fractions prepared from slices 1–5 by method 4, and gel 6 is the A_1 fraction prepared from full-thickness cartilage. Gels were stained with 0.1% Toluidine Blue.

reported in the present paper did not allow us to assess changes in the proportions of each band, investigations of bovine nasal (Roughley, 1977), human costal (Pearson & Mason, 1978) and human articular cartilage (Bayliss, 1982) have shown that, as the mobility increases, so the hydrodynamic size decreases and the keratan sulphate content increases. It therefore seems reasonable to expect an increase in the proteoglycans of highest mobility in the deeper layers of cartilage, and this would also be consistent with the gel-chromatographic changes observed.

A relationship between proteoglycan size and composition, similar to that described for different zones of cartilage, has also been observed for human articular cartilage with increasing age (Bayliss & Ali, 1978*a*,*b*; Roughley *et al.*, 1981), and it is noteworthy that in immature cartilage, which has much lower protein and keratan sulphate contents, the proportion of small-molecular-weight proteoglycan is considerably diminished (Bayliss & Ali, 1981). Furthermore, gel electrophoresis of preparations of immature cartilage shows the absence of band 3 and a smaller content of band 2 (Bayliss & Venn, 1980), which is further evidence in support of the electrophoretic results discussed above.

Unlike extracts from many other sources of hyaline cartilage, 4M-guanidinium chloride extracts of full-thickness human cartilage have been found to contain very few proteoglycan aggregates, and yet the proteoglycans in those extracts could still interact with hyaluronic acid (Bayliss & Ali, 1978*b*; Bayliss & Venn, 1980). These paradoxical findings were resolved by powdering the cartilage in liquid N_2 before extraction, after which the hyaluronic acid in the tissue was also extracted and most of the proteoglycans were purified in an aggregate form (Bayliss & Ali, 1978*a*). The results reported in the present paper show that the proteoglycans are still poorly aggregated even when the cartilage is sliced to 250μm. Analysis of the A_1 fractions and their gel-chromatographic profiles suggests that the smaller of the two proteoglycan pools is preferentially extracted from these slices and that the larger species is left in the tissue together with most of the hyaluronic acid. It was only by powdering the 250μm-thick slices that the full extent of aggregation could be realized. Although this method of tissue preparation has been used successfully in a number of investigations, it requires carefully controlled conditions (e.g. time and frequency of grinding) in order to avoid mechanical degradation of the polymers (Lohmander & Hjerpe, 1975; Bayliss & Venn, 1980; Bayliss, 1982). This makes powdering a relatively unsatisfactory method, and the less-disruptive technique of sectioning was found to give more consistent results (M. T. Bayliss, unpublished work). The lower aggregate content of extracts from 20μm-thick or 2μm-thick sections compared with extracts of powdered cartilage is probably a reflection of the increased extraction of proteoglycans, and as such is possibly more indicative of the true state of aggregation in each layer. These facts, together with the changes in structure and composition, suggest the following general interpretation of our results.

Proteoglycans have a large hydrodynamic size and they overlap and entangle with themselves and with the collagen (Hascall & Sajdera, 1970; Pasternak *et al.*, 1974; Reihanian *et al.*, 1979). Collagen in articular cartilage has a fine spacing (of the order of 100nm), and this is further subdivided by proteoglycans into pore sizes of less than 5nm (Maroudas, 1979). Diffusion of proteoglycans through these fine pores will be slowed down considerably because of this entanglement, and the larger proteoglycans will move more slowly than the smaller ones. Klein &

Meyer (1982) have shown that it takes several weeks for hyaluronic acid to move through connective tissues. The rate of diffusion is proportional to the inverse of the distance squared (Crank, 1975); thus extraction from 20 μm-thick slices will be 150 times faster than from 250 μm-thick slices. Since the same time was allowed for extraction from both slice sizes, diffusion properties alone could explain why a larger proportion of small proteoglycans was extracted in the latter case. Hyaluronic acid extraction, however, may depend more on disruption of the collagen network, which would result in less entanglement, although diffusion of these large molecules would also be very slow in thick slices. From a teleological point of view it is noteworthy that the surface of cartilage, which is in contact with synovial fluid and through which the proteoglycans are therefore most likely to be lost, has both the tightest collagen network and the largest proteoglycans.

In relation to degenerative joint disease, one of the chemical changes accompanying cartilage fibrillation is a preferential loss of keratan sulphate from the tissue (Mankin & Lippiello, 1971; Venn & Maroudas, 1977; Bayliss & Venn, 1980). Since the smaller proteoglycans have a higher keratan sulphate content, it is not surprising to find that these are lost first from the partially disrupted tissue. The mechanism need be no more than simple diffusion, though this does not of course exclude other processes being involved.

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