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Isolation of Proteoglycans from Human Articular Cartilage

By MICHAEL T. BAYLISS and S. YOUSUF ALI
Experimental Pathology Unit, Institute of Orthopaedics (University of London), Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middx. HA7 4LP, U.K.

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Proteoglycans were extracted from normal human articular cartilage of various ages with 4M-guanidinium chloride and were purified and characterized by using preformed linear CsCl density gradients. With advancing age, there was a decrease in high-density proteoglycans of low protein/uronic acid weight ratio and an increase in the proportion of lower-density proteoglycans, richer in keratan sulphate and protein. Proteoglycans at each age were also shown to disaggregate in 4M-guanidinium chloride and at low pH and to reaggregate in the presence of hyaluronic acid and/or low-density fractions. Osteoarthrotic-cartilage extracts had an increased content of higher-density proteoglycans compared with normal cartilage of the same age, and results also suggested that these were not mechanical or enzymic degradation products, but were possibly proteoglycans of an immature nature.

Proteoglycans have been extracted from a wide variety of cartilage sources by using various dissociative conditions such as 4M-guanidinium chloride, and shown to exist as high-molecular-weight complexes (Sajdera & Hascall, 1969; Rosenberg *et al.*, 1970; Tsiganos *et al.*, 1971; Hardingham & Muir, 1974; Strider *et al.*, 1975). Chondroitin sulphate and keratan sulphate are attached along a polypeptide backbone, and it is the variable substitution of these polysaccharides on the protein chain that gives rise to the large polydispersity observed in many preparations (Hascall & Sajdera, 1970; Tsiganos *et al.*, 1971). Variability in the amino acid composition of the protein chain has also been suggested as contributing to this polydispersity (Tsiganos *et al.*, 1971; Hardingham *et al.*, 1976). Recent evidence has indicated that most proteoglycans are present in cartilage linked to a hyaluronic acid backbone to form aggregates (Hardingham & Muir, 1972, 1973, 1974; Hascall & Heinegård, 1974*a*,*b*) and that these complexes are stabilized by a glycoprotein component (Hascall & Sajdera, 1969; Gregory, 1973; Heinegård & Hascall, 1974) and can be disaggregated in high-salt solutions and by low solution pH (Hascall & Sajdera, 1969; Hardingham & Muir, 1974). Age-related changes in the chemistry and size of the proteoglycans from pig articular cartilage have been reported (Simunek & Muir, 1972). However, similar studies on human articular cartilage have been limited to changes in crude proteoglycan extracts of mechanically homogenized tissue (Rosenberg *et al.*, 1965).

The results given here are part of an extensive investigation of a number of biochemical factors involved in the normal aging and osteoarthrotic processes associated with human articular cartilage (Bayliss, 1976). In the present paper, methods are described for extracting and studying proteoglycans from normal and diseased human articular cartilage. A preliminary report of part of this work has been published (Bayliss & Ali, 1976).

Experimental

Materials

All reagents were of analytical grade, except glucuronolactone and carbazole, and were supplied by BDH Chemicals, Poole, Dorset, U.K. Acetylacetone was redistilled (b.p. 133–134°C). Hyaluronic acid (human umbilical cord) was obtained from BDH, and Visking Tubing from Scientific Instruments Centre, London W.C.1, U.K.

Analytical methods

Uronic acid was determined by the Bitter & Muir (1962) modification of the carbazole method of Dische (1947) with glucuronolactone as standard. Hexosamine was determined by the procedure of Rondle & Morgan (1955) with glucosamine hydrochloride as standard. Samples were hydrolysed in 5M-HCl at 100°C for 4h. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparative methods

Extraction of proteoglycans. Human articular cartilage was obtained fresh from the operating theatre and either used immediately (within 1h) or stored at −20°C until required. Normal cartilage was

obtained from fracture of the neck of the femur specimens or amputation specimens.

Pathological cartilage was obtained from femoral heads resected for total hip replacement. The articular surface of each specimen was rinsed with cold sterile 0.9% NaCl before removal of full-thickness cartilage with a scalpel. Samples of cartilage were then prepared by one of two methods: (*a*) by dicing finely (2 mm^3) with a scalpel; or (*b*) by powdering in liquid N_2 with a Spex Freezer Mill (Spex Industries Inc., Metuchen, NJ, U.S.A.). In the latter case, when the cartilage samples weighed less than 0.1 g, the micro-vials provided with the instruments were used. A grinding time of 30 s for 1 g cartilage samples resulted in optimum extraction. Proteoglycans were extracted with 4 M-guanidinium chloride buffered at pH 4.0 or pH 5.8 with 0.1 M-sodium acetate buffer and pH 7.4 with 0.1 M-sodium phosphate buffer. Where a broad pH range was required, 0.1 M-Universal buffer was used (Ellis, 1961). Cartilage was suspended in ten times its weight of 4 M-guanidinium chloride in Universal bottles and rolled gently on a Dorchester Mixer (Raven Scientific Ltd., Haverhill, Suffolk, U.K.) at 4°C for 24 h. The extracts were centrifuged for 15 min at 1000*g* on a bench centrifuge, the supernatant was removed and the residue washed with a small volume of cold buffered 4 M-guanidinium chloride. The extract and washings were combined and adjusted to associative conditions by dialysis against 9 vol. of the appropriate 0.05 M buffer.

Preformed density gradients

A continuous linear density gradient of CsCl was obtained by using an instrument prepared in our workshop and described by Ali (1966). It is based on the original developed by Bock & Ling (1954) and on the design given by Shapland (1964). The density range for each gradient was 1.250–1.650 g/ml. The proteoglycan samples (1 ml) were layered on the surface of the gradients with a bulb pipette. Centrifugation was carried out in an M.S.E. Superspeed 50 centrifuge at 15°C in a 3 × 30 ml swingout rotor at 75000$g_{av.}$ for 5 or 10 h. After the centrifugation the gradients were fractionated by upward displacement and the fractions dialysed against 0.1 M-sodium phosphate buffer to remove CsCl. Both the formation of the linear gradient and the fractionation system were tested by including a red dye (Ponceau S) in preliminary experiments and also by measuring the density of each fraction by using a 100 μl constriction pipette. The gradients were linear at the time of formation, and are therefore represented as straight lines on the graphs.

Equilibrium density gradients

These were prepared by the method of Sajdera & Hascall (1969). Solid CsCl was added until a density of 1.69 g/ml was reached and the volume was made up to 18 ml with a solution of CsCl ($\rho = 1.69$ g/ml). This solution was then centrifuged to equilibrium at 95000$g_{av.}$ for 48 h at 20°C in an 8 × 25 ml angle rotor in an M.S.E. Superspeed 50 centrifuge. Fractions (1 ml) were collected as described for the preformed gradients and dialysed against a large volume of 0.05 M-phosphate buffer, pH 7.4.

Gel chromatography

A column (40 cm × 1.6 cm; Pharmacia) was packed with Sepharose 2B and suspended in 0.1 M-phosphate buffer, pH 7.4, containing 0.1 M-NaCl. Samples (0.5 ml) containing 200–300 μg of uronic acid were loaded on the column and eluted at 3 ml/h with a peristaltic pump. Blue Dextran 2000 and glucuronolactone were used to determine the void volume and total volume respectively.

Preparation of autolysis products

A sample (2 g) of normal cartilage was finely diced and incubated at 37°C for 48 h in 20 ml of 0.1 M-sodium acetate buffer, pH 5.0. Penicillin and streptomycin (Glaxo Laboratories Ltd., Greenford, Middx., U.K.) were included at 6 mg/ml and 10 mg/ml respectively. The charged species released into the incubation medium were precipitated by adding aq. 2.5% (w/v) cetyltrimethylammonium bromide until a flocculent precipitate was formed (Ali, 1969). The precipitate was left at 4°C overnight, then collected by centrifugation at 1000*g* for 15 min with a bench centrifuge, washed twice with 95% ethanol saturated with NaCl and dried at room temperature (22°C) in a desiccator. Samples used for gel chromatography were resuspended in the eluting buffer overnight at 4°C.

Results

Comparison of extraction procedures

When human articular cartilage was finely diced and extracted with guanidinium chloride, pH 7.4, by conventional techniques, even a 4 M solution would only extract 25% of the tissue uronic acid (Fig. 1*a*), and as a result the extraction procedures had to be evaluated.

Preliminary experiments demonstrated that the pH of the extracting media also influenced the efficiency of the extraction. At pH 5.0 40% of the tissue uronic acid was extracted, with less released at more acid or alkaline pH (Fig. 1*b*). This suggested that degradation by endogenous proteinases was occurring during extraction at room temperature and that this might also occur to a more limited extent at 4°C. Confirmation of this was obtained by purifying cartilage extracts obtained at pH 5.8 and pH 7.4 on equilibrium density gradients under 'associative' conditions (Hascall & Sajdera, 1969), starting density 1.69 g/ml. A sample of the bottom 2 ml from each gradient, containing 50–60% of the

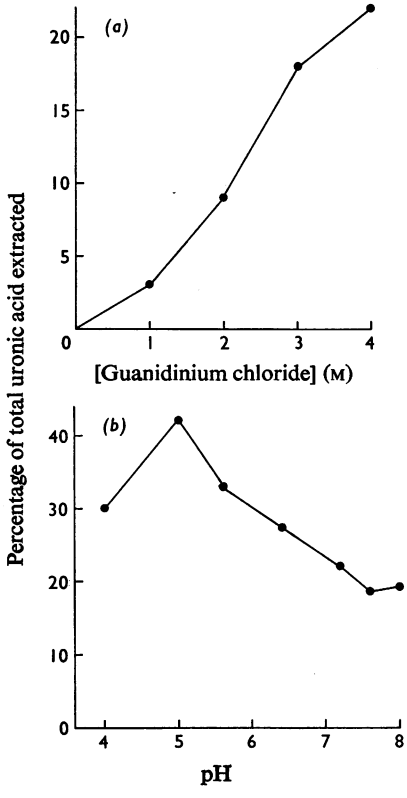

Fig. 1. *Effect of (a) guanidinium chloride concentration and (b) pH on the extraction of human articular cartilage*
(*a*) Full-thickness cartilage pieces from a normal femoral head (80-year-old patient) were extracted at room temperature for 24h with various concentrations of guanidinium chloride at pH7.4. (*b*) Similar cartilage from the same specimen was extracted with 4M-guanidinium chloride at various pH values at room temperature for 24h. The total uronic acid released was determined in each case.

uronic acid, was chromatographed on a column (40cm×1.6cm) of Sepharose 2B. Only a small proportion of the pH7.4 extract was excluded from the column, and the remainder was retarded as a peak with $K_{av.} = 0.14$ (Fig. 2*a*). However, the pH5.8 extract showed no excluded peak and all the material was retarded on the column ($K_{av.} = 0.26$). Autolysis products, which accounted for 60% of the tissue uronic acid, were completely retarded as a very broad peak with $K_{av.} = 0.47$ (Fig. 2*b*). In an attempt to improve the extraction and therefore obtain a representative sample of human articular-cartilage proteoglycans, the tissue was powdered in a Spex Freezer Mill in liquid N_2. This subsequently allowed the extraction of 80–85% of the tissue uronic acid with 4M-guanidinium chloride at both pH5.8 and pH7.4. The proteoglycans were purified on an associative density gradient as above and fractions (1–5), representing 70% of the uronic acid in each case, were almost totally excluded in the void volume of a Sepharose 2B column (Fig. 2*c*).

Preformed CsCl density gradients

Equilibrium density-gradient centrifugation is the most widely used method of purifying cartilage proteoglycans. Most proteoglycans are of very high density, and are therefore recovered as a mixture in the dense regions of the gradient. Further fractionation of the mixture is usually carried out on Sepharose 2B, but even by this method, the limits are such that molecular-weight variations within the excluded material cannot be fully analysed. Further, human articular cartilage could only be obtained in small amounts, particularly from pathological specimens. Therefore a method was developed that enabled the separation of proteoglycan species

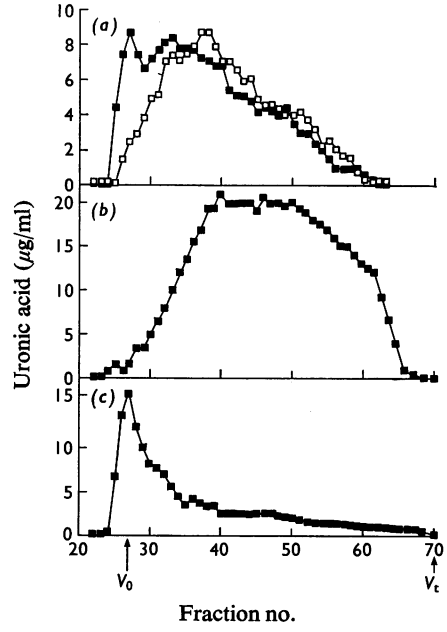

Fig. 2. *Gel chromatography on Sepharose 2B of (a) purified proteoglycans extracted from cartilage pieces at pH7.4 (■) and pH5.8 (□), (b) autolysis products and (c) purified proteoglycans extracted from cartilage powder at pH7.4*
Samples containing 200–300μg of uronic acid were applied to a column (40cm×1.6cm) of Sepharose 2B and eluted as described in the text. The uronic acid content of each 1ml fraction was determined. V_0 and V_t mark the void volume and total volume of the column respectively.

from small tissue samples without the lengthy purification on equilibrium density gradients.

Powdered cartilage samples of human femoral-head cartilage from 16- and 78-year-old patients were extracted with 4M-guanidinium chloride, pH7.4, and the extracts dialysed to associative conditions. A 1ml sample of each extract was applied to the surface of a preformed CsCl density gradient and centrifuged as described in the Experimental section. Because the gradients were identical, it was possible to compare directly the sedimentation patterns of different samples. After 10h centrifugation, most of the proteoglycans from the 16-year-old patient were present in fractions 1–4 (α^I) and fractions 5–10 (α^{II}) (Fig. 3*a*).

The cartilage extract from the 78-year-old patient had, however, distributed itself as one broad peak, fractions 1–10 (β) (Fig. 3*b*). This change in sedimentation pattern also corresponded to an increase in the hexosamine and protein contents of not only the proteoglycan species but also the total extract (Table 1).

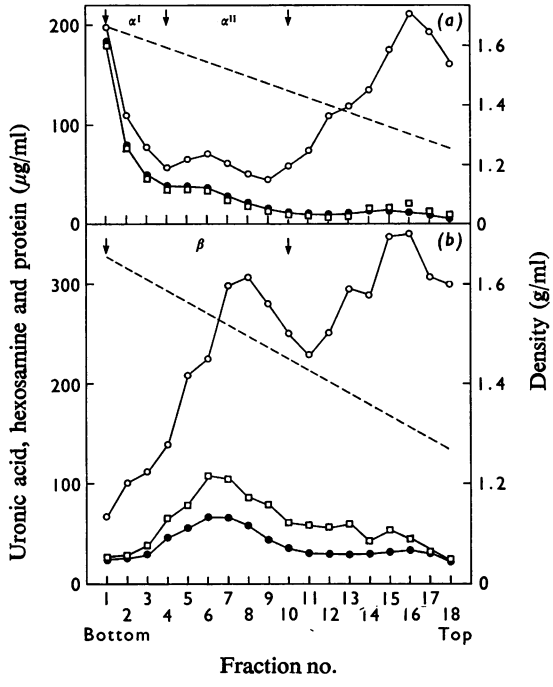

Fig. 3. *Sedimentation patterns of crude proteoglycan extracts from normal femoral-head cartilage after centrifugation on preformed CsCl density gradients; (a) 16-year-old and (b) 78-year-old patients*

A sample (1ml) of each of the above extracts in 0.4M-guanidinium chloride was layered on the surface of a preformed CsCl density gradient (----) and centrifuged for 10h at 75000$g_{av.}$ as described in the text. Fractions (1ml) were assayed for uronic acid (●), hexosamine (□) and protein (○).

Table 1. *Analysis of proteoglycans extracted with 4M-guanidinium chloride and fractionated on preformed CsCl density gradients*

Conditions of centrifugation and fractions analysed are as shown in Figs. 3 and 4.

Age and site	Fractions	Protein/uronic acid weight ratio	Hexosamine/uronic acid weight ratio
16 years (femoral head)	1–4, α^I	1.25	1.00
	5–10, α^{II}	2.70	1.10
78 years (femoral head)	1–10, β	3.78	1.43
5 years (talus)	1–4, α^I	1.31	1.10
	5–10, α^{II}	2.71	1.00
12 years (talus)	1–4, α^I	1.74	1.06
	5–10, α^{II}	2.72	1.08
64 years (talus)	1–10, β	3.60	1.56

Analysis of these gradients showed that the β species had a much higher hexosamine/uronic acid weight ratio than that of the α^I proteoglycans, which may reflect a higher keratan sulphate content. The greatest change with age, however, was in the protein/uronic acid weight ratio. This was up to three times higher in the β species than in the α^I species, and it was probably this increase that contributed most to the decreased rate of sedimentation. An increased protein/uronic acid weight ratio was also observed in the α^{II} species compared with the α^I component. Sedimentation patterns for specimens of various ages all had a very large peak of protein near the origin of the gradient which contained all the hydroxyproline present and is probably equivalent to the protein-rich fractions found on 'associative' equilibrium density gradients.

Further evidence for these age-related changes was obtained with samples of talus cartilage from 5-, 12- and 64-year-old patients. The extracted proteoglycans were centrifuged on three identical preformed gradients as described above (Fig. 4). Once again, the proteoglycan extract from the 64-year-old patient was characterized by the β species with its high hexosamine and protein content (Table 1). In the cartilage extract from the 5-year-old patient, the α^I species was predominant as expected, but the shoulder observed on the gradient of the 16-year-old patient was less prominent (Fig. 3*a*). However, the cartilage extract from the 12-year-old patient had a similar distribution to that from the 16-year-old patient, even though the α^I species accounted for slightly less of the total uronic acid.

Disaggregation of proteoglycans

An attempt was made to use the preformed density gradients to demonstrate disaggregation of the extracted proteoglycans. The assumption was made

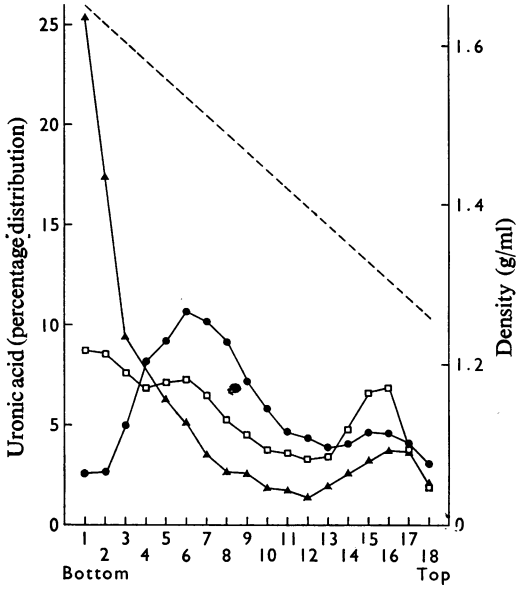

Fig. 4. *Sedimentation patterns of crude proteoglycan extracts of normal human talus cartilage, from 5- (▲), 12- (□) and 64- (●) year-old patients, after centrifugation on preformed CsCl (----) density gradients under associative conditions*

The conditions were the same as described in Fig. 3.

that if disaggregation took place in 4M-guanidinium chloride to give proteoglycan subunits and link components (Hascall & Sajdera, 1969), then on centrifugation the much larger subunits would move into the gradient far more quickly than the link and would therefore not reaggregate on leaving the 4M-guanidinium chloride zone.

Samples of the proteoglycan extracts from the 16-year-old patient in 0.4M-guanidinium chloride or in 4M-guanidinium chloride were layered on identical preformed gradients and centrifuged for 10h (Fig. 5*a*). The associated sample gave the normal α^{I} and α^{II} zones, but the α^{I} species was completely absent from the dissociated sample and was replaced by a broad peak with a distribution overlying that of an α^{II} species. This effect was also paralleled by a decrease in the protein/uronic acid weight ratio (Table 2). However, a lower protein/uronic acid weight ratio should have resulted in an increase in sedimentation rate if density was the only controlling factor. The most probable explanation, therefore, was a decrease in molecular weight due to disaggregation. This change in sedimentation rate was less apparent for proteoglycans extracted from normal cartilage obtained from aged individuals, because they were already sedimenting at a slower rate even in 0.4M-guanidinium chloride; however, Fig. 5(*b*) shows that under dissociative conditions there was a slightly decreased sedimentation rate.

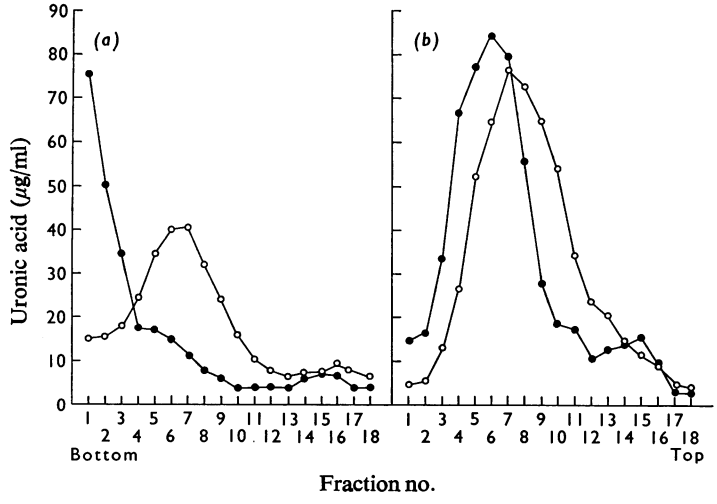

Fig. 5. *Distribution of proteoglycans extracted from normal femoral-head cartilage after centrifugation on preformed CsCl density gradients; (a) 16- and (b) 78-year-old patients*

Crude extracts in either 0.4M-guanidinium chloride (●) or 4M-guanidinium chloride (○) were layered on identical preformed gradients and centrifuged for 10h as described in the text.

Reaggregation of proteoglycans extracted from cartilage of a 16-year-old patient

To confirm that disaggregation had occurred, it was necessary to establish that the dissociated proteoglycans could be reassociated.

Fractions 1–10 and 11–18 from a dissociative gradient of proteoglycans extracted from the cartilage of a 16-year-old patient, as shown in Fig. 5(*a*), were combined. Both sets of fractions were adjusted to dissociative conditions by the addition of an equal volume of 8M-guanidinium chloride, pH7.4, and recombined in the proportions in which they occurred on the gradient, on the basis of the uronic acid content of each set of fractions. Another sample was also prepared at half this ratio, and also a control sample from which fractions 11–18 were absent. After dialysis to associative conditions, they were layered on three identical preformed gradients (Fig. 6*a*). Although the control sample did display a certain amount of α^{I} species, probably because fractions 1–10 were contaminated with linking components from the upper fractions, there was a definite peak at the α^{II} position. As the proportion of fractions 11–18 was increased in the system, so there was an increase in the proportion of α^{I} component and a concomitant decrease in the α^{II} species, reflecting reaggregation of the α^{II} component. Hardingham & Muir (1974) have demonstrated that hyaluronic acid is one of the aggregating factors, and the effect of hyaluronic acid from human umbilical cord on the above aggregating system is shown in Fig. 6(*b*). The control sample showed the sedimentation profile for disaggregated proteoglycans from the 16-year-old patient. Hyaluronic acid at a concentration of 0.1% (based on uronic acid content) had only a slight effect, but 1% hyaluronic acid resulted in a sedimentation profile very similar to that of the α^{I} species. Once again, this most probably resulted from an increase in molecular size.

Table 2. *Analysis of the associated and dissociated proteoglycan species from 16- and 78-year-old patients*
Conditions of centrifugation and fractions analysed are as shown in Fig. 5.

Fractions	Protein/uronic acid weight ratio
16-year-old patient	
1–4, α^{I}	0.98
1–10, α^{II}	0.58
78-year-old patient	
1–4, $\beta_{associated}$	3.3
1–10, $\beta_{associated}$	5.5
1–12, $\beta_{dissociated}$	5.2

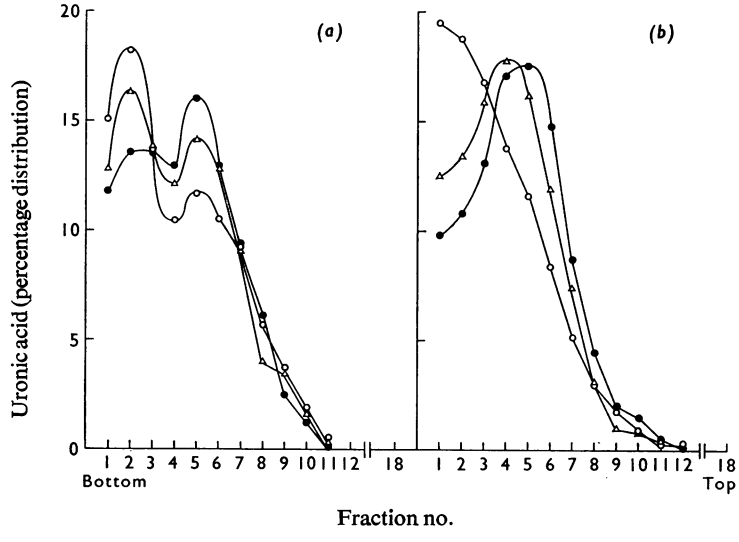

Fig. 6. *Recombination of disaggregated proteoglycans from the cartilage of a 16-year-old patient with (a) low-density fractions from a dissociative gradient and (b) hyaluronic acid*
(*a*) Fractions 1–10 from the dissociative gradient shown in Fig. 5(*a*) were recombined with the low-density fractions 11–18 from the same gradient. ●, Fractions 1–10 alone; △, fractions 1–10 and 11–18 recombined in half the proportions in which they occurred on the gradient; ○, fractions 1–10 and 11–18 recombined in the full gradient proportions (based on uronic acid content). (*b*) ●, Fractions 1–10 alone; △, fractions 1–10+0.1% hyaluronic acid; ○, fractions 1–10+1% hyaluronic acid. The gradient conditions were identical with those of Fig. 3.

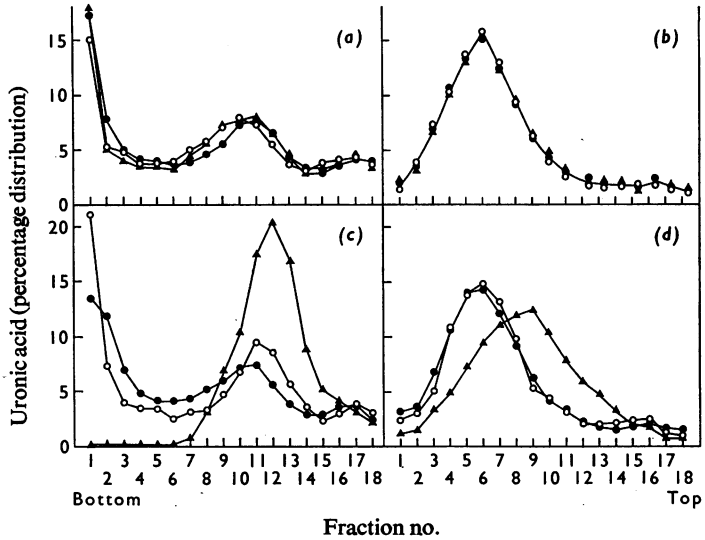

Fig. 7. *Effect of pH on extraction (a and b) and on aggregation (c and d)*
Cartilage samples were extracted at pH4.0 (▲), pH5.8 (○) and pH7.4 (●) and then reassociated at pH7.4; (*a*) 16- and (*b*) 78-year-old patients. For (*c*) and (*d*) reassociation was carried out at the pH of extraction; thus pH4.0 (▲), pH5.8 (○) and pH7.4 (●); (*c*) 16- and (*d*) 78-year-old patients. The extracts from the 16-year-old patient were centrifuged for 5h at 75000$g_{av.}$ and those from the 78-year-old for 10h at 75000$g_{av.}$ Gradients were the same as for Fig. 3.

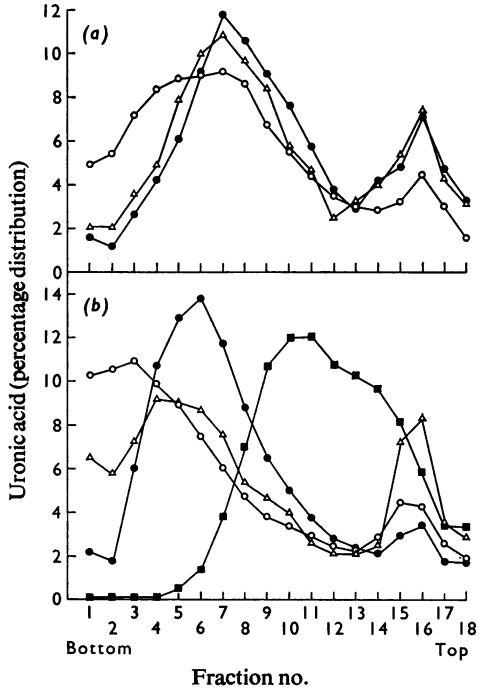

Fig. 8. *Centrifugation of proteoglycan extracts from osteoarthrotic cartilage in preformed CsCl density gradients*
Crude associated extracts were centrifuged on preformed gradients as described in the text. (*a*) Three samples of type IV cartilage from different areas of an osteoarthrotic femoral head (80-year-old patient). (*b*) Two samples of type IV cartilage (○) and (△) from an osteoarthrotic femoral head and an extract of normal femoral head cartilage (●) (60-year-old patient). Autolysis products of the normal specimen are also shown (■).

Effect of pH on aggregate formation

Sajdera & Hascall (1969) and Hardingham & Muir (1974) demonstrated that the proportion of aggregates in an extract depended on solution pH. Figs. 7(*a*) and 7(*b*) show the results of an experiment to determine the effect of extraction pH on aggregate formation. Cartilage samples were extracted at pH4.0, 5.8 and 7.4 and then dialysed to 'associative' conditions at pH7.4. The extracts from the 16-year-old patient were centrifuged for 5h instead of 10h to separate clearly the α^{I} and α^{II} components. There was the same proportion of α^{I} to α^{II} components at each pH, indicating the same extent of aggregation. Similarly, the extracts from the 79-year-old patient give the typical β peak after 10h centrifugation. To test the effect of pH on aggregation, each extract was dialysed to its appropriate pH (Figs. 7*c* and 7*d*). Once again proteoglycans from the 16-year-old patient, aggregated at pH5.8 and pH7.4, gave essentially the same distribution, although the pH5.8 sample seemed to have a slightly greater proportion

of α^{I} species. However, the pH4.0 extract consisted entirely of the α^{II} species, identical with the results obtained in 4M-guanidinium chloride. Proteoglycans from the 78-year-old patient, aggregated at pH4.0, also showed the shift to lower density that was observed in 4M-guanidinium chloride. These results therefore appeared to show a dissociation of the aggregates at pH4.0.

Preformed density-gradient centrifugation of pathological proteoglycans

Cartilage obtained from pathological specimens rarely exceeded 100mg wet wt. and was always of the residual type IV osteoarthrotic cartilage described by Ali & Bayliss (1974, 1975). Samples of the various cartilage extracts were fractionated on preformed gradients as described above, and a normal cartilage extract of approximately the same age was always centrifuged at the same time to act as a control. Fig. 8 shows the distribution of proteoglycans from three specimens of cartilage (type IV) removed from different areas of an osteoarthrotic femoral head (80-year-old patient). Although two of the samples gave the typical β pattern characteristic for their age, the third specimen had an increased concentration of higher-density proteoglycans. In another specimen, both samples of type IV cartilage had an increase in higher-density proteoglycans compared with the normal control (Fig. 8*b*). This increased population of higher-density species was unlikely to be the result of degradation by endogenous enzymes, as shown by the much slower sedimentation rate of autolysis products isolated from normal cartilage of the same age. It seems likely that the pathological cartilage contained a greater proportion of juvenile proteoglycans compared with age-matched controls. The results also emphasize the variable nature of osteoarthrotic-cartilage proteoglycans, even those isolated from the same specimen but from different sites.

Discussion

The poor extraction of human cartilage 'chips' agrees with the results of Altman *et al.* (1973) who found that 6M-LiCl only extracted 30% of the uronic acid. The low extraction at various pH values, however, is in marked contrast with the finding that 3M-guanidinium chloride, between pH3.8 and 8.4, solubilized approx. 85% of bovine nasal-cartilage hexuronate (Hascall & Sajdera, 1969). The inefficient extraction is probably due to the greater thickness of the human, as compared with the bovine cartilage pieces, and to a denser collagen network in the former (i.e. there was a physical barrier to extraction). It is also possible that the human proteoglycans are more firmly associated with the collagen network, especially in the specimens from elderly patients.

A representative sample of proteoglycans from human cartilage was obtained by grinding the samples at the temperature of liquid N_2. Lohmander & Hjerpe (1975) also used this technique to obtain extracts of rib and epiphysial cartilage, and their results suggested that prolonged grinding caused some degradation of the proteoglycans, evident as an increase in material eluted at the total volume of a Sepharose 2B column. The grinding times were 10–15 times as long as those used by us and, as a consequence, local heat generation would have been extreme. Gel chromatography of proteoglycans from powdered human cartilage revealed that, as with other cartilages, a large proportion of them are in a high-molecular-weight form. Further, no effect of pH was observed on the yield of proteoglycan from powdered cartilage extracts, which suggests that proteolysis might have occurred in the thicker cartilage pieces used in the preliminary experiments.

By using linear preformed CsCl density gradients under dynamic conditions, we have resolved some of the aging and pathological changes in human articular cartilage. Although interpretation of different rates of sedimentation as a molecular size difference is only rigorously applicable to substances of identical composition and conformation, changes in the rate of sedimentation, together with changes in the chemical composition of proteoglycans, can provide useful information about their structure.

Comparison of the sedimentation profiles of cartilage extracts from specimens of various ages, together with changes in their protein/carbohydrate ratio, confirmed that with advancing age there was an increase in proteoglycans of higher protein and keratan sulphate content. Rosenberg *et al.* (1965) first observed this change in the composition of human cartilage proteoglycans when they isolated them by the method of Gerber *et al.* (1960). Although it is not possible to compare the products obtained by Rosenberg *et al.* (1965) directly with those obtained in the present study, because of differences in the isolation procedures, they showed that compared with children (0–12 years old), the adults (17–73 years old) had an increased protein, hexose and sialic acid content of both the protein–polysaccharide light (PP-L) and protein–polysaccharide heavy (PP-H) fractions.

Disaggregation of the human proteoglycan species on the preformed gradients should depend on a large decrease in size and change in protein/carbohydrate weight ratio, by analogy with results obtained on animal cartilages. Thus when most of the dissociated proteoglycans (α^{II}) from the 16-year-old patient sedimented at a slower rate than the associated α^{I} species, this was probably the result of a decrease in molecular size. The dissociated protein was seen as an increase in the low-density fractions, 17 and 18, confirming results obtained with equilibrated gradients. Complications arising from density shifts of

the components of proteoglycans in CsCl/guanidinium chloride equilibrium density gradients have been observed by Mashburn *et al.* (1974). Their results show that the guanidinium ion does not follow the CsCl gradient, but forms a reverse gradient, thus slightly decreasing the density gradient. By confining any changes in the guanidinium chloride concentration in our experiments to the medium in which the sample was applied, which was only 5% of the total gradient volume, alterations in the sedimentation patterns due to interaction with the counter-ions were eliminated. The evidence also suggests that the small amount of α^{II} component observed in the associated extracts of cartilage from juvenile patients is a mixture of disaggregated α^{I} species and the β species.

Experiments carried out on the α^{II} proteoglycan species confirmed that there was a specific interaction of low- and high-density material, in agreement with the original experiments of Sajdera & Hascall (1969). Tsiganos *et al.* (1971) and Hardingham & Muir (1974) also observed similar results by gel chromatography, albeit using more specific fractions. The reaggregation of disaggregated proteoglycans with hyaluronic acid is also consistent with the observations of Hardingham & Muir (1972, 1974) and Hascall & Heinegård (1974*a*,*b*). Hyaluronic acid, at very low concentrations, has been found to affect the rate of sedimentation of other macromolecules in a centrifugal field, by acting as a molecular sieve (Laurent & Pietruskiewicz, 1961; Laurent *et al.*, 1963). However, the increased sedimentation rate in the presence of hyaluronic acid, observed here, excludes any possibility of physical entrapment and suggests instead that a specific interaction occurred. Although the same amount of uronic acid was extracted from pig laryngeal cartilage at various pH values, the ability to form aggregates was greatest in the pH4.5 extract (Hardingham & Muir, 1974). The extraction pH did not affect the ability of the various human proteoglycan extracts to form aggregates. However, the pH at which the extracts were associated did affect the proportion of aggregates and emphasizes the importance of ionic linkages in aggregate formation (Hascall & Heinegård, 1974*a*,*b*; Heinegård & Hascall, 1974; Hardingham *et al.*, 1976).

As a result of the work of Hardingham & Muir (1974) and Hascall & Heinegård (1974*a*,*b*) a model has been described that accounts for the polydispersity of proteoglycan subunits by proposing that there is a decrease in size of the chondroitin sulphate-binding region in the proteoglycans of high protein content and also a lower extent of substitution with chondroitin sulphate chains (Hardingham *et al.*, 1976). Therefore, if the high protein content of the proteoglycans from elderly patients is the result of an increase in the population of proteoglycans of lower molecular weight, with a high protein/carbohydrate ratio, then either (i) the aggregates in mature cartilage are smaller than those in immature cartilage or (ii) the aggregates are of the same size, in which case the hyaluronic acid backbone in mature cartilage would have to be longer to accommodate more subunits of smaller size. Hascall & Heinegård (1974*a*,*b*) have shown that the size of the hyaluronic acid molecule determines the aggregate size, and this may be the case in human articular cartilage.

Mankin & Lipiello (1971) postulated that the chondrocytes of osteoarthrotic cartilage reverted to a chrondroblastic phase and started producing an immature matrix. This hypothesis was based on the appearance of a matrix rich in chondroitin 4-sulphate. Similarly, the work of McDevitt *et al.* (1974, 1977) also confirmed that proteoglycans with a higher galactosamine/glucosamine ratio were present in osteoarthrotic cartilage from dogs. Even more important were their observations that the changes were not limited to the site of lesion, but were present throughout the cartilage of the affected joint. The altered sedimentation patterns observed here for some human osteoarthrotic specimens also suggest an increased proportion of juvenile proteoglycans in the extracts. Similarly, elevated activities of alkaline phosphatase and cathepsin B1 (Ali & Bayliss, 1974, 1975) in osteoarthrotic and immature articular cartilage also tend to support the hypothesis that in osteoarthrosis, articular cartilage may revert to a juvenile state.

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