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Articular-Cartilage Proteoglycans in Aging and Osteoarthritis

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The composition of macroscopically normal hip articular cartilage obtained from dogs of various ages was studied. Pieces of cartilage with signs of degeneration were studied separately. In normal aging, the extraction yield of proteoglycans decreased; the keratan sulphate content of extracted proteoglycans increased and the chondroitin sulphate content decreased. The extracted proteoglycans were smaller in the older cartilage, mainly owing to a decrease in the chondroitin sulphate-rich region of the proteoglycan monomers. The hyaluronic acid-binding region and the keratan sulphate-rich region were increased and the molar concentration of proteoglycan probably increase with increasing age. The degenerated cartilage had higher water content and the proteoglycans, as well as other tissue components, gave higher yields. The proteoglycan monomers from the degenerated cartilage were smaller than those from normal cartilage of the same age, and hence had a smaller chondroitin sulphate-rich region and some of the molecules also appeared to lack the hyaluronic acid-binding region. Increased proteolytic activity may be involved in the process of cartilage degeneration.

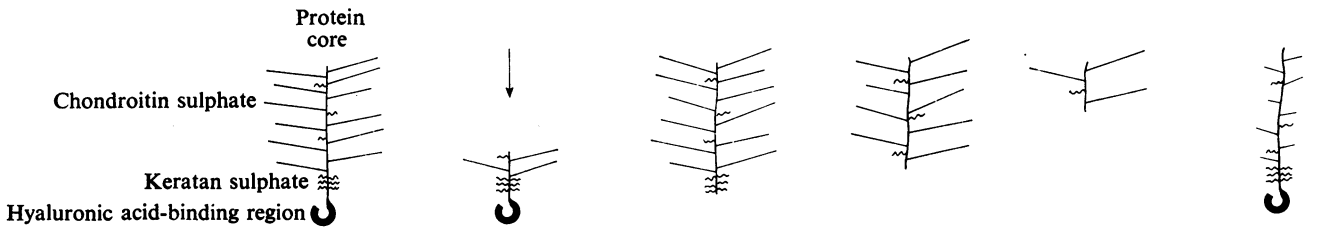
Articular cartilage contains few cells and an abundance of surrounding extracellular matrix, which consists mainly of collagen and proteoglycan. The main components contributing to the elasticity of the articular cartilage are the proteoglycans (for references see Kempson, 1975), which are composed of a central protein core to which a large number of highly negatively charged polysaccharide chains of chondroitin sulphate and keratan sulphate are covalently attached. At one end, the hyaluronic acid-binding region (see Fig. 1), about one-third of the protein core forms a globular structure (Heinegård & Hascall, 1974*a*) to which few or no polysaccharide chains are attached. This region is capable of interacting specifically with hyaluronic acid. Several individual proteoglycan monomers can bind to one hyaluronic acid molecule to form large aggregates. In normal cartilage a large proportion (80–90%) of the proteoglycans are able to form aggregates. The major portion of the keratan sulphate chains and a few chondroitin sulphate chains are attached to the keratan sulphate-rich region (see Fig. 1) (Heinegård & Axelsson, 1977). The remaining 90% of the chondroitin sulphate chains and some keratan sulphate chains are attached to a third portion of the protein core, the chondroitin sulphate-rich region (see Fig. 1) (Heinegård & Axelsson, 1977) The chondroitin sulphate-rich region is probably essential for the resilience of the proteoglycans, because it contains a large number of negatively charged groups.

The elasticity of cartilage is largely related to the content and the structure of the proteoglycans in the matrix (Harris *et al.*, 1972; Scott, 1973, 1975). Kempson *et al.* (1971) showed that the elasticity of osteoarthritic cartilage is decreased. It is therefore likely that the composition of the proteoglycans has changed.

Primary oesteoarthritis is usually a disease of old age in both man and animals. In contrast, osteoarthritis that is secondary to disease or trauma often occurs in young and middle-aged individuals. In secondary osteoarthritis, the macroscopic and histological changes in the joint cartilage are similar to those found in osteoarthritis. Hip dysplasia with secondary osteoarthritis is common in certain breeds of dogs; such dogs could therefore be a readily available source for studying changes of the proteoglycan composition in degenerated cartilage. A prerequisite for better understanding of the changes of cartilage composition in osteoarthritis, however, is a knowledge of the normal changes with age.

In pig articular cartilage the glucosamine/galactosamine ratios increased with age (Šimůnek & Muir, 1972). This implies that the content of keratan sulphate compared with chondroitin sulphate is relatively higher in the articular cartilage from the older animal.

It has been suggested that degradation of the proteoglycans is an important step in the development of osteoarthritis. Accordingly Ali & Evans (1973) reported an increased activity of cathepsins in osteoarthritis with a concomitant loss of uronic acid,



Size of proteoglycan monomer	Normal	Small	Normal (−)	Normal (−)	Small	Small
Size of chondroitin sulphate	Normal	Normal	Normal	Normal	Normal	Small
Chondroitin sulphate content (galactosamine, uronic acid)	Normal	Low	Normal (+)	High	High	Low
Keratan sulphate content (glucosamine)	Normal	High	Normal (+)	Low	Low	High
Protein content (hyaluronic acid-binding region)	Normal	High	Low	Low	Low	High
Ability to aggregate	+	+	−	−	−	+
Buoyant density	Normal	Low	Normal (+)	Normal (+)	Normal (+)	Low

Fig. 1. *Structure and composition of proteoglycan monomers after conceivable modifications*
The structure proposed for proteoglycans from aging cartilage is indicated with an arrow. (The normal structure is to the left, with all parameters indicated as normal.)

probably because of degradation of the proteoglycans. McDevitt *et al.* (1973) reported an increased ratio of galactosamine/glucosamine in proteoglycans isolated from osteoarthritic knee-joint cartilage of dogs. The osteoarthritis had been induced by cutting the anterior cruciate ligament in the knee joint. Altman *et al.* (1973) have reported decreased uronic acid/protein ratios in proteoglycans from osteoarthritic cartilage.

In summary, available data indicate that the proteoglycan structure is changed in osteoarthritis, although seemingly contradictory results have been obtained.

Work on the structure of cartilage proteoglycans indicated that protein, keratan sulphate and chondroitin sulphate are asymmetrically distributed in the proteoglycan molecules (see Fig. 1) (Heinegård & Hascall, 1974*a*; Heinegård & Axelsson, 1977). Therefore changes in the relative proportions of the polysaccharides and protein can be used as markers of changing size of the proteoglycans, and also to indicate the mechanism behind these changes. Direct measurements of the size of the proteoglycans will give supporting information.

The aim of the present investigation was primarily to establish whether there are changes in the quantity and structure of the proteoglycans of aging articular cartilage, and secondarily to establish whether an altered proteoglycan structure in the degenerated cartilage of osteoarthritis (when compared with normal cartilage of the same age) could explain the decreased elasticity of the cartilage.

Materials

Fresh articular cartilage was obtained within 5 min after death from the femoral heads of ten dogs, aged 4.5 months–13 years (eight German Shepherd dogs and two Afghan Hounds, both the latter aged 5 years). From eight of the dogs, both apparently normal cartilage and degenerated cartilage was obtained. Cartilage was considered degenerated when it was discoloured, had lost its normal glossiness or was eroded. In all dogs with degenerated cartilage, hip dysplasia was the cause of osteoarthritis. In three dogs (age 6, 8 and 12 months) normal cartilage was taken from animals with osteoarthritis of one hip only, and in these cases the normal cartilage was taken from the normal contralateral hip joint. From five animals (aged 0.5, 5, 5, 8 and 10.5 years) cartilage with normal appearance was taken from the same joint as the degenerated cartilage. From one of these dogs, aged 6 months, apparently normal cartilage was obtained both from a joint that had focal cartilage degeneration and from the apparently normal contralateral joint. The youngest dog (aged 4.5 months) had subluxation of both hip joints, and 19 days before it was killed it had been operated on. Pelvic osteotomy was performed on one side in an effort to minimize subluxation. The operation was a failure and actually caused degenerative changes to appear in the joint cartilage. All the cartilage in the contralateral joint had a normal appearance. One dog, aged 17 months, had normal cartilage in both hip joints and a sample was taken only from one joint. Only degenerated cartilage could be obtained from one dog, aged 13 years, which had advanced osteoarthritis of both hip joints.

All samples of degenerated cartilage were taken from the small semilunar area dorsal to ligamentum teres, which is a major weight-bearing part of the femoral head. Cartilage samples from normal joints were taken from the same location on the joint surface. In some cases apparently normal cartilage was taken from the dorsal aspect of the femoral head, which contained areas showing degeneration. Since all cartilage samples were prepared from the dorsal and most weight-bearing area of the femoral head, it was considered that differences in the structure of the proteoglycans would not be due to variations in the site from which the macromolecules were prepared.

In all cases, the cartilage was classified as being either normal (whitish-blue with glossy surface) or degenerated. Four grades of degeneration were recognized: (*a*) discoloured cartilage with glossy surface; (*b*) fibrillated cartilage with loss of normal glossiness; (*c*) eroded cartilage; (*d*) severely eroded cartilage adjacent to defects with denuded subchondral bone.

Cartilage samples were dissected, wiped clean with a paper napkin, cut into small pieces (approx. 2 mm in diam.) and frozen in liquid N_2. The frozen samples were then immediately put into a stainless-steel cylindrical chamber (diam. 10 mm, depth 32 mm), precooled in liquid N_2. A stainless-steel cylindrical piston (diam. 9.5 mm, height 61 mm), also precooled in liquid N_2, was then fitted into the chamber and the cartilage was powdered by applying a pressure of 196 MPa to the piston for 2 s. The cartilage powder was stored at −30°C until extraction.

Methods

Extraction

Samples (9.1–230.8 mg) of cartilage powder from the various dogs were extracted in 3 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, for 24 h at 3°C (Hascall & Sajdera, 1969; Heinegård, 1972). Extracts were separated from residues by centrifugation at 21 700*g* ($r_{av.}$ 8.6 cm) for 20 min in a Sorvall RC 2-B centrifuge with the SE-12 rotor at 4°C. The residues were resuspended in 0.5 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, and again centrifuged at 22 700*g* ($r_{av.}$ 9.0 cm) for 20 min. The supernatants were added to the respective extracts discussed above. This procedure was

repeated twice to assure complete separation of extracts from residues.

Associative-density-gradient centrifugation (Hascall & Sajdera, 1969; Heinegård, 1972)

The concentration of guanidinium chloride in the extracts was brought to about 0.4M by dialysis of the extracts against 9 vol. of 0.05M-sodium acetate, pH 5.8, for 24h at 3°C. The densities were subsequently adjusted to 1.61–1.62g/ml by the addition of solid CsCl. The solutions were centrifuged in an MSE preparative ultracentrifuge in a 10×10ml aluminium angle rotor at 34000rev./min (83000*g*, $r_{av.}$ 6.46cm) for 60h at 12°C. Immediately after centrifugation the tubes were placed vertically in a stand and frozen by immersion in liquid N_2. The frozen tubes were then sawn in two (by using a guide) to separate the bottom 40% (by vol.) of the gradient (fraction A1) from the top (fraction A2). Densities of the bottom and the top fractions were measured by pycnometry with a 250μl constriction pipette.

Chemical methods

Protein contents of fraction A1 were determined by the micro-procedure of Lowry *et al.* (1951) after the samples had been dialysed against sodium acetate and water. Uronic acid in both fractions A1 and A2 were measured by the procedure of Bitter & Muir (1962), but with one-tenth volumes. Hydroxyproline contents of cartilage samples were determined as described by Stegemann & Stalder (1967) after hydrolysis of samples in 6M-HCl in sealed tubes for 24h at 100°C.

Keratan sulphate and chondroitin sulphate contents of extracts and residues were determined on samples digested with papain, and the glycosaminoglycans were isolated by ion-exchange chromatography as described by Axelsson & Heinegård (1975). The samples were then hydrolysed in 6M-HCl for 8h in sealed tubes at 100°C and glucosamine and galactosamine were separated and determined with an automatic amino acid analyser. Galactosamine contents of the glycosaminoglycan fractions are proportional to the contents of chondroitin sulphate, whereas glucosamine contents of the glycosaminoglycan fractions are approximately proportional to the contents of keratan sulphate. Hyaluronic acid, however, also contains glucosamine, but, since the quantities of hyaluronic acid in cartilage is very small (Hardingham & Muir, 1973; Hascall & Heinegård, 1974), its contribution to the glucosamine content is minimal and will be disregarded. Glycosaminoglycans of extracts and residues were calculated as the sum of glucosamine and galactosamine in the glycosaminoglycan fractions.

The proportions of aggregates and monomers in fraction A1 were determined by gel chromatography on Sepharose 2B. The excluded peaks represent the aggregates (Heinegård, 1972).

To determine representative differences in the size distribution of the proteoglycan monomers in fraction A1, the ability of the monomers to interact with hyaluronic acid was first inhibited by reduction and alkylation of the preparations (Heinegård, 1977). Such a treatment destroys the tertiary structure and the function of the hyaluronic acid-binding region by breaking the disulphide bridges. The samples were then chromatographed on Sepharose 2B, to yield the elution profiles of the proteoglycan monomers. The size distribution of the chondroitin sulphate side chains of the proteoglycans in fraction A1, was determined after the samples had been digested with papain (5μg/mg of proteoglycan) in 0.05M-sodium phosphate buffer/5mM-cysteine/5mM-EDTA, pH 6.5 at 65°C, for 3h. The samples were then chromatographed on Sephadex G-200 (Wasteson, 1971).

Gel chromatography

Sepharose 2B and Sephadex G-200 gel chromatography was performed on columns (about 140cm×0.6cm) eluted with 0.5M-sodium acetate, pH 7.0, at a constant temperature of 4°C. Constant flow of eluent (1.5ml/h) through the columns was obtained by using a constant-flow-rate infusion pump equipped with a 50ml plastic syringe. Fractions (0.89ml) were collected with a time-controlled fraction collector and analysed for uronic acid content by an automated version of the carbazole method (Heinegård, 1973).

Calculations

The analytical values obtained with normal and osteoarthritic cartilage from the same individuals were compared by Student's *t* test for paired observations. The youngest dog (aged 4.5 months) was not included in the *t* test, as it had bilateral subluxation of the hip. Linear-regression correlation coefficients for values from all cartilage, excluding the degenerated cartilage from the operated joint of the youngest dog, were calculated by the method of least squares. Age was correlated to the chemical data obtained both with the normal and the degenerated cartilage. A Student's *t* test (Rao, 1965) was performed on each regression line from the normal cartilages to test the hypothesis that the line does not deviate from horizontal, i.e. that the chemical parameters remain constant through all age groups.

Results and Discussion

Normal cartilage

Composition. Glycosaminoglycans (per wet weight or per hydroxyproline content of whole cartilage) showed a small decrease with increasing age, although

the linear correlation coefficients were low (Table 1). Hydroxyproline (per wet weight of cartilage) showed no significant variation with age (Table 1). The keratan sulphate content of cartilage showed a significant increase with age when expressed as percentage of glycosaminoglycans (correlation coefficient 0.89, $P<0.001$), but also when related to hydroxyproline content or wet weight of cartilage (Table 2). The relative keratan sulphate content increased more in the cartilage from the youngest dogs, but as discussed the oldest contained more keratan sulphate.

The amounts of keratan sulphate compared with chondroitin sulphate in cartilage increase with age (Mathews & Glagov, 1966; Šimůnek & Muir, 1972), and our data corroborate these findings. However, the absolute amount of keratan sulphate in the articular cartilage increases with age and there is only a minor decrease in contents of total cartilage glycosaminoglycans with age.

Extraction. In the present work proteoglycans were extracted with guanidinium chloride without the proteinase inhibitors used by Oegema *et al.* (1975). It should be noted, however, that all procedures were done in the cold and in a direct sequence. Therefore it is unlikely that any degradation of the macromolecules occurred during extraction. Cartilage proteoglycans prepared from guanidinium chloride extracts with and without proteinase inhibitors have the same structure and composition (D. Heinegård, unpublished work). It should also be pointed out that

Table 1. *Composition of normal and osteoarthritic cartilage*

Extraction yields and tissue contents of glycosaminoglycans are related to wet weight and hydroxyproline contents.

Normal cartilage	Age (months)	Hydroxyproline per wet wt. of cartilage (%)	Extraction yield [% of polysaccharide hexosamine (extract/tissue)]	Glycosaminoglycan contents: Glycosaminoglycan hexosamine/wet wt. cartilage (μg/mg)			Glycosaminoglycan hexosamine/hydroxyproline in tissue (μg/μg)		
				Extract	Residue	Tissue	Extract	Residue	Tissue
	4.5*	5.18	89.3	34.4	3.9	38.3	0.662	0.079	0.741
	4.5	2.72	80.6	16.8	4.1	20.9	0.625	0.150	0.775
	6	4.84	81.9	22.6	5.0	27.6	0.467	0.102	0.569
	6*	3.43	82.4	15.4	3.2	18.6	0.449	0.095	0.544
	8	4.20	75.9	12.7	3.9	16.7	0.303	0.095	0.397
	12	2.54	76.8	7.2	2.2	9.3	0.283	0.084	0.367
	17	4.19	76.2	21.7	6.8	28.5	0.517	0.161	0.678
	60*	4.28	67.8	10.7	5.2	15.9	0.251	0.121	0.372
	60*	3.36	69.9	17.9	7.5	25.4	0.532	0.227	0.759
	96*	3.66	65.1	10.4	5.6	15.9	0.210	0.165	0.435
	126*	3.28	56.6	10.6	7.9	18.4	0.320	0.245	0.566
Mean	36	3.79	74.8	16.4	5.0	21.4	0.420	0.139	0.564
Correlation coefficient		−0.20	−0.93	−0.45	0.69	−0.28	−0.52	0.77	−0.18
P (regression line)		>0.4	<0.001	<0.2	<0.02	<0.4	<0.1	<0.01	>0.4
Osteoarthritic cartilage Classified group									
a	4.5	3.45	72.1	6.4	2.5	9.0	0.188	0.073	0.261
a	6	3.14	82.0	16.5	3.6	20.0	0.523	0.115	0.637
b	8	4.45	88.8	18.6	2.3	20.9	0.417	0.052	0.469
b	12	1.87	78.9	8.1	2.1	10.2	0.426	0.115	0.541
c	60	2.76	70.6	6.4	2.7	9.1	0.232	0.098	0.330
c	60	3.07	72.4	7.0	2.7	9.7	0.227	0.086	0.313
c	96	2.40	73.6	11.1	3.9	15.0	0.462	0.166	0.628
b	126	3.32	63.6	7.7	4.5	12.2	0.233	0.134	0.367
d	156	2.87	75.6	17.4	5.5	22.9	0.603	0.195	0.797
Mean	59	3.04	75.3	11.0	3.3	14.3	0.368	0.115	0.483
Correlation coefficient		−0.14	−0.67	−0.09	0.85	0.10	0.04	0.77	0.24
P (normal versus osteoarthritic)		<0.05	<0.025	<0.3	<0.01	<0.2	>0.4	>0.2	>0.4

* Apparently normal cartilage from joint with arthritic changes.

Table 2. *Composition of normal and osteoarthritic cartilage*
Contents of chondroitin sulphate and keratan sulphate are shown in extracts, extraction residues and tissue.

Normal cartilage	Age (months)	Keratan sulphate/chondroitin sulphate (glucosamine as % of polysaccharide hexosamine)			Keratan sulphate contents: Glycosaminoglycan glucosamine/wet wt. cartilage (μg/mg)			Keratan sulphate contents: Glycosaminoglycan glucosamine/hydroxyproline in tissue (μg/μg)		
		Extract	Residue	Tissue	Extract	Residue	Tissue	Extract	Residue	Tissue
	4.5*	5.7	7.4	5.9	1.95	0.30	2.26	0.038	0.005	0.043
	4.5	8.9	4.3	8.0	1.50	0.16	1.66	0.055	0.007	0.063
	6	12.9	5.9	11.7	2.92	0.30	3.22	0.061	0.005	0.066
	6*	9.3	6.1	8.7	1.43	0.20	1.63	0.041	0.007	0.048
	8	11.2	8.4	10.5	1.41	0.34	1.75	0.034	0.007	0.041
	12	17.6	9.9	15.8	1.27	0.20	1.47	0.050	0.009	0.059
	17	22.7	14.9	20.9	4.92	1.15	6.07	0.118	0.023	0.141
	60*	26.2	18.3	23.7	2.81	0.93	3.74	0.066	0.022	0.087
	60*	28.7	20.4	26.2	5.12	2.49	6.68	0.152	0.047	0.199
	96*	24.2	19.9	22.7	2.51	1.11	3.62	0.068	0.030	0.098
	126*	39.9	26.8	34.2	4.19	2.15	6.34	0.127	0.066	0.193
Mean	36	18.9	12.9	17.1	2.73	0.76	3.49	0.074	0.021	0.094
Correlation coefficient		0.89	0.94	0.89	0.50	0.78	0.64	0.59	0.90	0.71
P (regression line)		<0.001	<0.001	<0.001	<0.2	<0.01	<0.05	<0.1	<0.001	<0.02
Osteoarthritic cartilage Classified group										
a	4.5	6.8	9.8	7.7	0.45	0.25	0.70	0.013	0.007	0.020
a	6	13.6	16.9	14.2	2.24	0.61	2.85	0.070	0.020	0.090
b	8	6.7	7.6	6.8	1.25	0.18	1.43	0.027	0.004	0.030
b	12	20.6	17.1	19.9	1.65	0.36	2.00	0.088	0.020	0.107
c	60	24.3	21.9	23.6	1.56	0.59	2.15	0.057	0.021	0.078
c	60	30.6	21.9	28.2	2.13	0.59	2.72	0.068	0.020	0.088
c	96	24.3	22.7	23.8	2.69	0.91	3.60	0.111	0.038	0.149
b	126	32.9	32.9	32.8	2.56	1.45	4.01	0.077	0.043	0.120
d	156	32.8	27.8	31.6	5.67	1.58	7.25	0.199	0.054	0.252
Mean	59	21.4	19.8	21.0	2.24	0.72	2.97	0.079	0.025	0.104
Correlation coefficient		0.83	0.86	0.85	0.79	0.94	0.86	0.73	0.93	0.80
P (normal versus osteoarthritic)		>0.4	<0.025	>0.4	<0.1	<0.2	<0.2	>0.4	>0.4	>0.4

* Apparently normal cartilage from joint with arthritic changes.

any proteolytic cleavage of the proteoglycan molecules, resulting in peptide liberation, would tend to decrease the protein content of fraction A1, in contrast with observations for aging cartilage (see below).

The proportion of the proteoglycans that could be extracted with 4M-guanidinium chloride decreased with increasing age (Table 1). Similar results have been obtained with pig articular (Šimůnek & Muir, 1972) and bovine tracheal cartilage (Larsson & Heinegård, 1975). The decreased ease of extraction of proteoglycans with increasing age cannot be readily explained at present since the structural or functional difference between extracted and residual proteoglycans is obscure. It should be noted (see below) that the proteoglycans that were not extracted contained relatively less keratan sulphate compared with the molecules extracted at each age (Table 2), which may indicate structural differences. Further, the content of keratan sulphate of extracted proteoglycans showed a regular increase with age, the linear regression correlation coefficient being 0.89 and $P<0.001$. This finding agrees with results of others (Mathews & Glagov, 1966; Šimůnek & Muir, 1972). The relative proportion of keratan sulphate in the glycosaminoglycans of the residues also increased with increasing age (Table 2), all values, however, being lower than those of the extracted proteoglycans of the same age.

Associative-density-gradient centrifugation. The content of uronic acid in the fraction A1 is proportional to the chondroitin sulphate and therefore

Table 3. *Composition of normal and osteoarthritic cartilage*

Relative distribution and contents of chondroitin sulphate (uronic acid) and protein are shown in fractions from associative CsCl-density-gradient centrifugation.

Normal cartilage	Age (months)	Chondroitin sulphate/protein (uronic acid/protein in fraction A1)	Uronic acid (% of dry wt.) Fraction A1	Uronic acid (% of dry wt.) Fraction A2	Protein in fraction A1 (% of dry wt.)	Uronic acid in fraction A1 (% of total uronic acid in gradient)
	4.5*	1.24	16.5	9.0	13.2	69
	4.5	1.75	24.0	8.1	13.7	84
	6	1.25	14.0	9.2	11.2	68
	6*	1.35	24.4	6.5	18.1	86
	8	1.60	30.4	8.6	19.0	77
	12	0.89	11.8	7.6	13.2	77
	17	1.10	22.5	8.2	20.4	83
	60*	0.98	13.9	9.9	14.2	65
	60*	0.72	20.3	7.7	28.1	83
	96*	1.00	20.3	8.9	20.3	68
	126*	0.82	17.3	9.1	21.0	61
Mean	36	1.16	19.6	8.4	17.5	75
Correlation coefficient		−0.65	−0.20	0.38	0.51	−0.59
P (regression line)		<0.05	>0.4	<0.3	<0.2	<0.1
Osteoarthritic cartilage Classified group						
a	4.5	1.44	15.9	6.8	11.1	58
a	6	1.69	23.0	5.1	13.6	78
b	8	1.93	26.6	4.4	13.8	85
b	12	1.11	20.0	6.3	18.1	86
c	60	1.71	21.9	6.9	12.8	66
c	60	0.77	12.0	6.7	15.4	61
c	96	1.21	23.2	7.7	19.3	81
b	126	1.08	17.0	5.0	15.8	78
d	156	0.78	17.7	10.3	22.7	62
Mean	59	1.30	19.7	6.6	15.8	73
Correlation coefficient		−0.64	−0.45	0.67	0.65	−0.50
P (normal versus osteoarthritic)		<0.01	>0.4	<0.005	<0.3	<0.4

* Apparently normal cartilage from joint with arthritic changes.

to the proteoglycan content of this fraction. The chondroitin sulphate (uronic acid) content of the fraction A1 expressed as percentage of the total chondroitin sulphate in the gradient decreased with increasing age of the cartilage (Table 3). Proteoglycans that have a lower buoyant density in a CsCl gradient usually are smaller and have a relatively higher protein content and a lower chondroitin sulphate content (Heinegård, 1977; Rosenberg *et al.*, 1976). Therefore the distribution of proteoglycans in the gradient (Table 3) indicated that the proportion of proteoglycans with higher protein content (i.e. smaller molecules) increases with age. Table 3 also shows that the protein content of fraction A1 increases with increasing age, indicating a higher protein content of the proteoglycans. Since the protein content of the proteoglycans is largely dependent on the hyaluronic acid-binding region, it is likely that the relative content of the latter increases with increasing age. The relative uronic acid content of fractions A1, however, showed only a minor decrease with increasing age. Such a seemingly contradictory result could simply be explained by the fact that the chondroitin sulphate chains (uronic acid) are by far the major component of the proteoglycans and that small differences in the chondroitin sulphate contents may be technically difficult to discern.

The spacing of chondroitin sulphate chains along the polysaccharide-binding region of the protein core is considered to be similar for proteoglycans of all sizes (Heinegård & Hascall, 1974*b*; Heinegård, 1977; Heinegård & Axelsson, 1977; Thyberg *et al.*, 1975). Therefore the number of chondroitin sulphate chains is probably determined by the relative length of this region of the core. It is assumed in the present work that degenerated or aging cartilage has the same spacing of chondroitin sulphate chains as the normal cartilage. The decreasing uronic acid/

protein ratio (Table 3) of fraction A1 with increasing age, then, indicates that the chondroitin sulphate-binding region of the molecule [containing little protein (Heinegård & Axelsson, 1977)] becomes smaller with increasing age, assuming that the hyaluronic acid-binding region is intact (see below).

The glucosamine/galactosamine ratio (keratan sulphate/chondroitin sulphate) of the extracted proteoglycans increased with increasing age (Table 2). The major portion of the keratan sulphate is located in the keratan sulphate-enriched region between the hyaluronic acid-binding region and the chondroitin sulphate-enriched region (Heinegård & Axelsson, 1977). It appears, then, that proteoglycans from older cartilage contain a relatively higher proportion of the keratan sulphate-enriched region.

The increased content of the hyaluronic acid-binding region and of the keratan sulphate-enriched region, compared with the chondroitin sulphate-enriched region of the proteoglycans from the older cartilage, suggests that the size of the molecules decreases with increasing age (cf. Fig. 1).

Gel chromatography. All cartilage fraction-A1 preparations contain variable proportions of proteoglycan aggregates and non-aggregated proteoglycan monomers (Hascall & Sajdera, 1969; Heinegård, 1972; Hascall & Heinegård, 1974). Sepharose 2B gel chromatography (Heinegård, 1972) is the technically most simple method to determine the proportion of non-aggregated proteoglycans in fraction A1 isolated from cartilage (Fig. 2). Only minor differences can be seen between the various chromatograms. The proportion of the non-aggregated monomer proteoglycans (included peak, Fig. 2), however, is somewhat larger in the very youngest cartilage. The results suggest that the capability of the proteoglycans to bind to hyaluronic acid is not significantly changed in the older cartilage and therefore it is likely that the same proportion of the molecules from all age groups contain the hyaluronic acid-binding region.

The size of the proteoglycan monomers in fraction A1 was determined by Sepharose 2B chromatography. To prevent aggregation the proteoglycans were reduced and alkylated in 4M-guanidinium chloride before chromatography on Sepharose 2B (Heinegård, 1977; Fig. 3). The advantage of this reduction technique compared with conventional CsCl-density-gradient centrifugation is that it accounts for all the monomers in fraction A1, whereas the density gradient selects larger monomers (Heinegård, 1977). The older the cartilage the more retarded were the proteoglycan monomers eluted (Fig. 3). Therefore the size of the proteoglycan monomers gradually decreased with increasing age of the cartilage.

Fractions A1 were digested with papain and chromatographed on Sephadex G-200 to reveal the

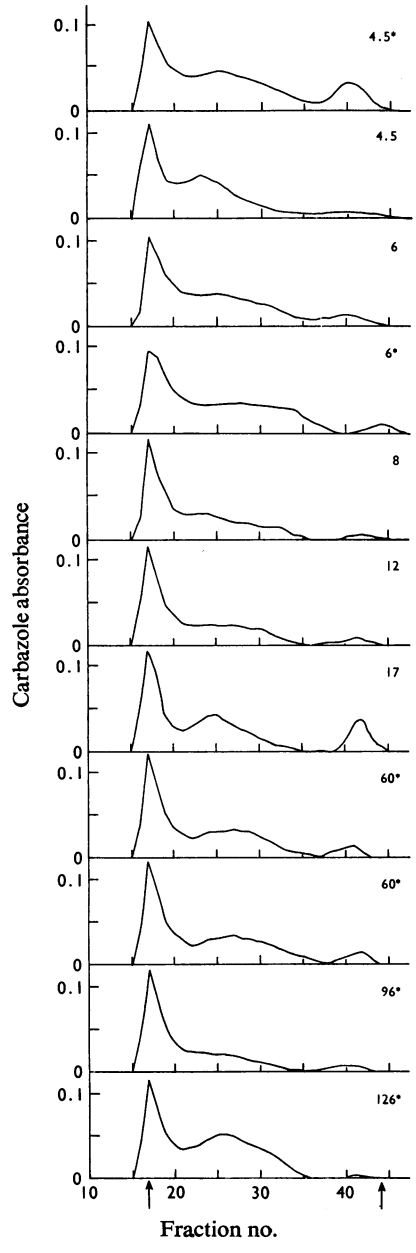


Fig. 2. *Sepharose 2B column (1400mm×6mm) chromatograms of fraction A1 from the associative-density-gradient centrifugation from normal cartilage*

The size of the peaks is corrected so that the area under the void volume peak is constant. The void volume and total volume are indicated by arrows. The age of the dog (in months) is given on each part of this and subsequent Figures. *Normal cartilage obtained from joint with degenerative changes.

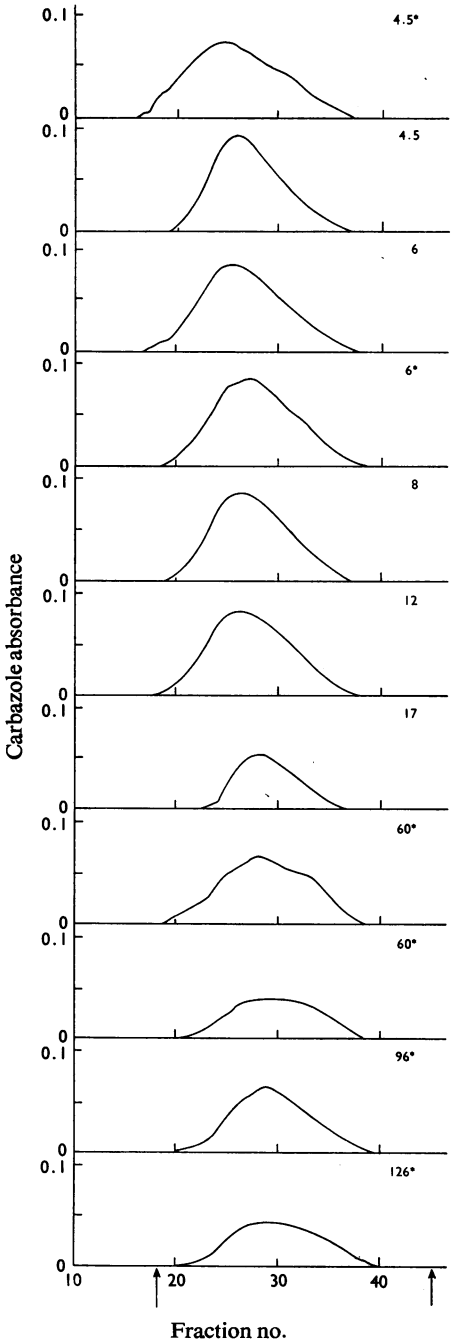


Fig. 3. *Sepharose 2B column* (1420mm×6mm) *chromatograms of reduced and alkylated fraction A1 from the associative-density-gradient centrifugation from normal cartilage*

The patterns represent the proteoglycan monomers. The void volume and the total volume are indicated by arrows. *Normal cartilage obtained from joint with degenerative changes.

size distribution of the chondroitin sulphate chains. The elution patterns indicate that there is only a very small decrease in the size of the chondroitin sulphate chains with increasing age of the cartilage (Fig. 4). Therefore it is likely that the decreasing size of the proteoglycan monomers can be attributed to a decreasing size of the proteoglycan protein core in the older cartilage, rather than to a decreasing size of the chondroitin sulphate side chains.

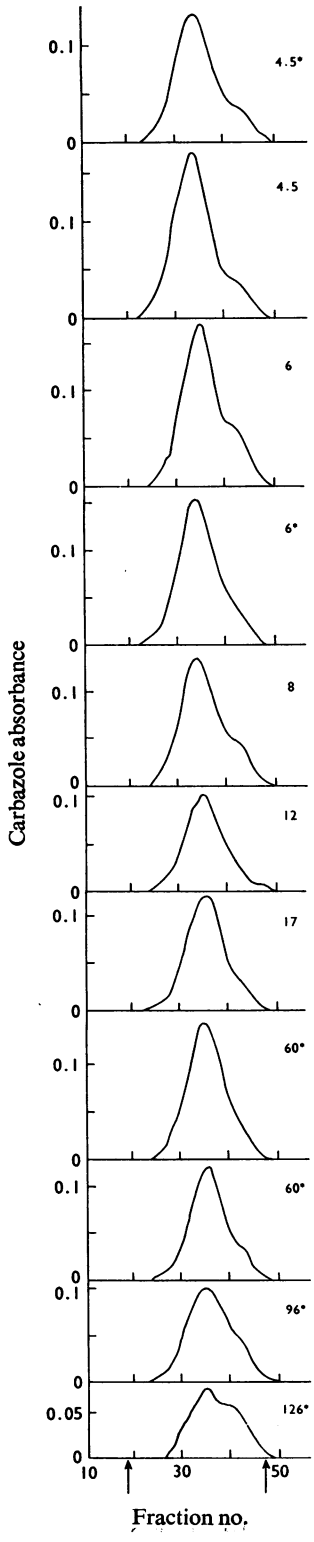
Degenerated cartilage in osteoarthritis

The large variations in proteoglycan composition in aging cartilage stresses the importance of using controls of comparable age when degeneration of cartilage is studied. Therefore all parameters of degenerated cartilage discussed below are compared with the same parameters of normal cartilage of the same age.

Composition. The total glycosaminoglycan content (expressed per wet weight of cartilage) was somewhat lower in degenerated than in normal cartilage of the same age (Table 1), although not statistically significant. It has been suggested (Matthews, 1953; Bollet *et al.*, 1963; Bollet & Nance, 1966; Mankin & Lipello, 1970) that such changes occur in osteoarthritis. The contents of total glycosaminoglycans when expressed per hydroxyproline contents, however, showed no difference when compared with normal cartilage, presumably owing to significantly lower ($P<0.05$) contents of hydroxyproline per wet weight of the degenerated cartilage (Table 1). The lower hydroxyproline contents of degenerated cartilage are indicative of a higher water content in osteoarthritic cartilage. These data corroborate findings of others (Bollet & Nance, 1966; McDevitt & Muir, 1976; Mankin & Zarins-Trasher, 1975). Bollet & Nance (1966) and Mankin & Lipello (1970) also found that the collagen content related to tissue dry weight was the same in normal and degenerated cartilage. Similar results with increasing water content of the cartilage that contained smaller amounts of proteoglycans were observed in experiments by Lipshitz *et al.* (1976). They used enzymic treatment or extraction of cartilage with salt solutions to remove the proteoglycans.

Degenerated cartilage contained more keratan sulphate with increasing age (Table 2). Accordingly the keratan sulphate/chondroitin sulphate ratios increased, as did the keratan sulphate contents, both when related to wet weight and to hydroxyproline contents of cartilage. These changes are similar to, and statistically not significantly different from, the variations observed in the normal cartilage (Table 2).

Extraction of degenerated cartilage. The extraction yields of proteoglycans from the degenerated cartilage varied similarly to those observed for normal cartilage: decreasing yields with increasing age. There was, however, a significantly greater extraction



yield in the degenerated compared with normal cartilage ($P<0.025$) (Table 1). Similar results have been obtained by others (McDevitt *et al.*, 1973; Brandt, 1974). Their procedures, however, were not designed to minimize effects of tissue proteinases and of other degradative enzymes. It is possible, then, that some degradation of the cartilage matrix may have obscured their results. Keratan sulphate content (expressed as percentage of total glycosaminoglycan content in the extracts) did not differ appreciably in degenerated compared with the normal cartilage (Table 2). The relative keratan sulphate content of the residues after extraction, however, was significantly higher ($P<0.025$) in the degenerated cartilages. In contrast, the keratan sulphate content of the residues, when related to the hydroxyproline content, was not significantly different in the degenerated cartilage (Table 2).

Associative CsCl-density-gradient centrifugation. The proportion of the total proteoglycans recovered in fraction A1 from the degenerated cartilages had a tendency to decrease with increasing age of the cartilage (Table 3),

The composition of fraction A1 showed increasing protein and decreasing uronic acid contents with increasing age (correlation coefficients 0.65 and −0.45 respectively). Such changes were also noted for the normal cartilage (see above). The uronic acid/protein ratios of fraction A1 from the degenerated cartilages, however, were significantly higher ($P<0.01$) than the ratios from the normal cartilage. Another difference was the significantly lower ($P<0.005$) uronic acid contents of fraction A2 from the degenerated cartilage (Table 3). Since the proportion of the total uronic acid that was recovered in fraction A2 is the same for normal and degenerated cartilage, it is likely that the differences observed reflect the presence of a large proportion of non-proteoglycan material in the extracts of the degenerated cartilage.

Gel chromatography. Sepharose 2B chromatography of fraction A1 from the degenerated cartilage showed a smaller proportion of aggregated proteoglycans, and therefore more monomers, than did the elution patterns of proteoglycans from normal cartilage (Fig. 5). Such a difference could be due to absence of the hyaluronic acid-binding region from the proteoglycan monomers from degenerated cartilage (see below).

Fig. 4. *Sephadex G-200 column (1465 mm × 6 mm) chromatograms of the papain-digested fraction A1 of the associative-density-gradient centrifugation from normal cartilage*
The patterns represent the chondroitin sulphate side chains. The void volume and the total volume are indicated by arrows. *Normal cartilage obtained from joint with degenerative changes.

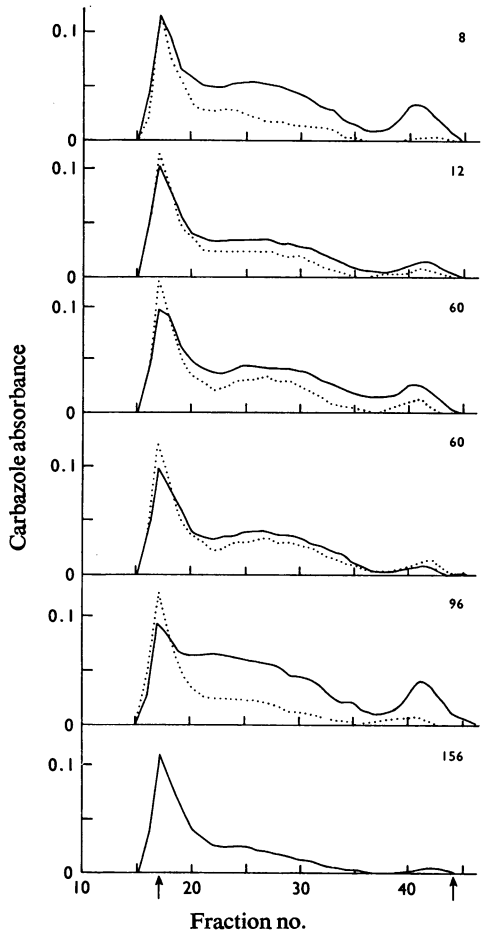


Fig. 5. *Sepharose 2B column* (1400*mm*×6*mm*) *chromatograms of the fraction A1 from the associative density-gradient centrifugation from osteoarthritic cartilage* (——) *and from normal cartilage from the same individual* (····)

The size of the peaks is corrected so that the area under the void volume peak is constant. The void volume and the total volume are indicated by arrows.

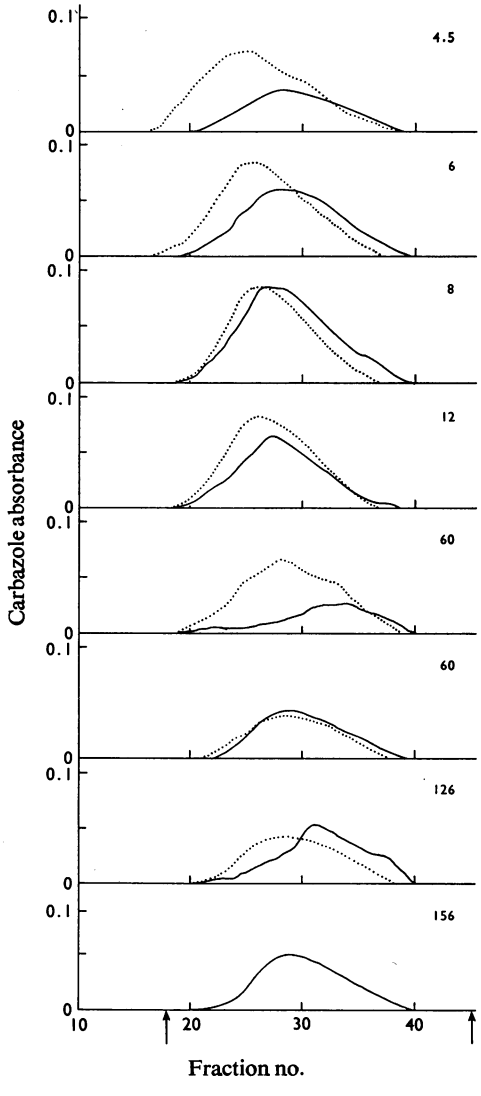


Fig. 6. *Sepharose 2B column* (1420*mm*×6*mm*) *chromatograms of reduced and alkylated fraction A1 from the associative-density-gradient centrifugation from osteoarthritic cartilage* (——) *and from normal cartilage from the same individual* (····)

The patterns represent the proteoglycan monomers. The void volume and the total volume are indicated by arrows.

The proteoglycan monomers from the degenerated cartilages were smaller than those from normal cartilage, as shown by the more retarded peaks after Sepharose 2B chromatography (Fig. 6). Sephadex G-200 chromatography of papain digests of the proteoglycans was used to monitor the size of the chondroitin sulphate chains. The chromatograms (Fig. 7) indicated that the chondroitin sulphate chains in the proteoglycans (fraction A1) from the degenerated cartilage were not appreciably different from those of the control cartilages of the same age.

Owing to shortage of material only the samples from degenerated cartilage indicated in Figs. 5, 6 and 7 could be chromatographed.

The degenerated cartilage was macroscopically graded into four subclasses. There were, however, too few animals in each subgroup to allow for any

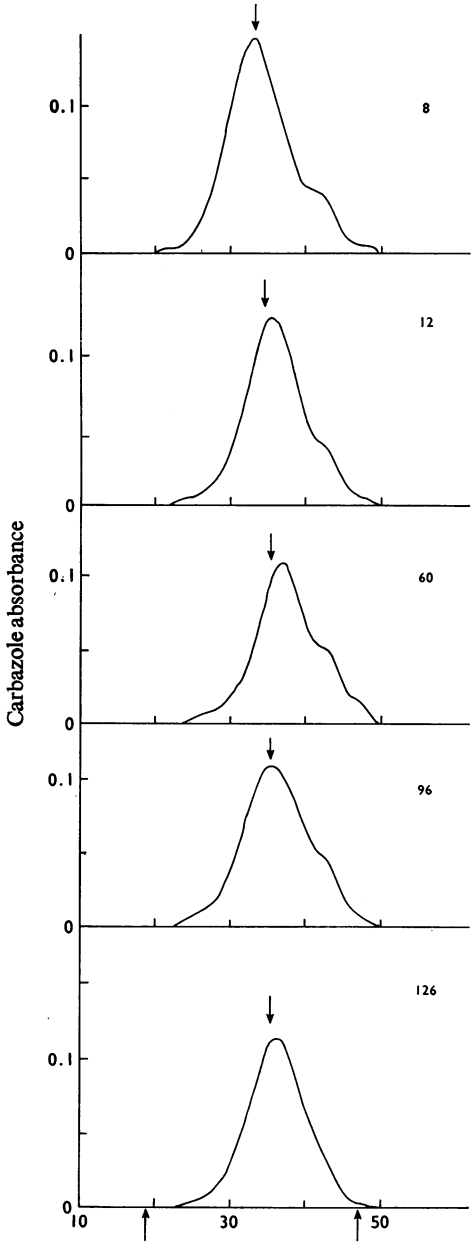


Fig. 7. *Sephadex G-200 column (1465 mm × 6 mm) chromatograms of the papain-digested fraction A1 of the associative-density-gradient centrifugation from osteoarthritic cartilage*
The patterns represent the chondroitin sulphate side chains. The arrows above the peaks indicate the elution peak of normal cartilage chondroitin sulphate chains (from Fig. 4). The void volume and the total volume are indicated by arrows.

attempt to statistically correlate the degree of degeneration to the structural changes of the proteoglycans.

General Discussion

The elastic properties of cartilage are determined by the three-dimensional organization and fixation of the charged groups (i.e. mainly the chondroitin sulphate chains) and therefore by the proteoglycan structure, rather than only by their quantity. Therefore the proteoglycan structure was studied; the investigation was, however, for technical reasons limited to the extractable proteoglycans. The asymmetric distribution of protein and glycosaminoglycan chains in cartilage proteoglycans is indicated in the current model of proteoglycan monomer structure shown in Fig. 1 (normal). The contents of protein, keratan sulphate and chondroitin sulphate in the proteoglycans can be utilized as markers, to discern whether the proteoglycans are altered in aging or in disease, as indicated in the protocol shown in Fig. 1. Minor and probably non-detectable differences are listed within parentheses. The size of the keratan sulphate chains will not be discussed because the normal chain size is not well established (Heinegård & Axelsson, 1977) and there is no documented technique for measuring the size of the keratan sulphate chains.

Articular-cartilage proteoglycans contain the keratan sulphate-rich region (D. Heinegård & I. Axelsson, unpublished work). The results of the various analyses show that with increasing age of the cartilage (1) the size of the proteoglycan monomers decreases, (2) the average size of the chondroitin sulphate chains does not change appreciably, (3) the chondroitin sulphate content, measured as contents of galactosamine and uronic acid, decreases, (4) the keratan sulphate content increases, (5) the protein content increases, (6) the ratio of aggregates to monomers, which can be used as an indication of the capability of the monomers to form aggregates or used as an indicator for the presence of hyaluronic acid-binding region, does not change and (7) the proportion of proteoglycans of lower buoyant density increases.

Thus the changing composition with age indicates that the proteoglycans undergo a gradual decrease in size. The change is probably due to a diminishing chondroitin sulphate-rich region, whereas the hyaluronic acid-binding region and the keratan sulphate-rich region remain comparatively constant. This change is consistent with the decreasing elasticity of the older cartilage since the chondroitin sulphate-rich region, which is most important for the elasticity of the cartilage, is decreasing in size. Results indicating similar changes in articular cartilage proteoglycan structure with age have been obtained by Strider *et al.* (1976).

Whether the changes observed are a result of an increased degradation of normally synthesized proteoglycans or a result of the biosynthesis of altered proteoglycans monomers is not answered by the present investigation, although some comments can be made. In the event of an increased degradation of proteoglycans, one would expect to find small-molecular-size chondroitin sulphate-rich peptides derived from the chondroitin sulphate-rich region. In the aging cartilage, no such fragments could be demonstrated. It is still possible, however, that such fragments are removed from the tissue very rapidly, and therefore not detectable.

All cartilage proteoglycan molecules, regardless of size, probably contain the keratan sulphate-rich region (Heinegård, 1977), whereas the chondroitin sulphate-rich region may vary. Therefore the keratan sulphate content of a cartilage is probably a better indication of the number of proteoglycan molecules (i.e. their molar concentration) in the tissue. Bearing this in mind it is noteworthy that the absolute keratan sulphate content of cartilage increases with age. Thus the molar concentration of proteoglycan monomers in the tissue probably increases with increasing age, contrary to what would be expected from the chondroitin sulphate contents. This observation may indicate either that the synthesis of proteoglycan monomers is increased or that their degradation and removal is decreased.

Only one animal was used in all but one age group and so no estimate could be made of variations within each group. It is unlikely, however, that the differences observed are due to variations between individuals. The trend with changes to a smaller proteoglycan with significantly higher keratan sulphate and protein contents with increasing age indirectly indicates that individual variations within each age group are of lesser importance.

All the parameters studied for the degenerated osteoarthritic cartilage showed similar variations with age as in the normal cartilage. However, a few differences were noted. The degenerated cartilage had a higher water content (wet weight divided by hydroxyproline contents), than normal cartilage of the same age. The extraction yield was significantly higher from osteoarthritic cartilage, and as indicated by the density gradient centrifugation more non-proteoglycan material was extracted. Such a result may imply a less tight network of the intercellular matrix, with less interactions between the various components. The reason may be an increased activity of proteinases, shown to be present in increased amounts in osteoarthritic cartilage by Ali & Evans (1973). Support for such a hypothesis derives from the other differences noted. The proteoglycans in fraction A1 from the degenerated cartilage had significantly higher uronic acid/protein ratios, although the molecules were smaller and contained at least as much keratan sulphate. Further, gel chromatography of fraction A1 showed lower contents of proteoglycan aggregates in the degenerated tissues. The data thus indicate that an increased proportion of the proteoglycan did not contain the hyaluronic acid-binding region.

In conclusion, the data indicate that the proteoglycans from the degenerated cartilage were smaller than those from the same-age normal cartilage, and had partially lost their ability to bind to hyaluronic acid and form aggregates.

The changes of the proteoglycan structure in the degenerated cartilage were of different nature, compared with those occurring in normal aging articular-cartilage proteoglycans, regardless of whether the normal cartilage was obtained from a normal joint or from joints with areas with degeneration. It is possible, then, that the processes leading to degeneration are focal.

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References

Ali, S. Y. & Evans, L. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1494–1498

Altman, R. D., Pita, J. C. & Howell, D. S. (1973) *Arthritis Rheum.* **16**, 179–185

Axelsson, I. & Heinegård, D. (1975) *Biochem. J.* **145**, 491–500

Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

Bollet, A. J. & Nance, J. L. (1966) *J. Clin. Invest.* **45**, 1170–1177

Bollet, A. J., Handy, J. R. & Sturgill, B. C. (1963) *J. Clin. Invest.* **42**, 853–859

Brandt, K. D. (1974) *Biochem. J.* **143**, 475–478

Hardingham, T. E. & Muir, H. (1973) *Biochem. Soc. Trans.* **1**, 282–284

Harris, E. D., Jr., Parker, H. G., Radin, E. L. & Krane, S. M. (1972) *Arthritis Rheum.* **15**, 497–503

Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4232–4241

Hascall, V. C. & Sajdera, W. S. (1969) *J. Biol. Chem.* **224**, 2384–2396

Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 181–192

Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201

Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1990

Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979

Heinegård, D. & Hascall, V. C. (1974*a*) *J. Biol. Chem.* **249**, 4250–4256

Heinegård, D. & Hascall, V. C. (1974*b*) *Arch. Biochem. Biophys.* **165**, 427–441

Kempson, G. E. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 277–305, North-Holland Publishing Co., Amsterdam and Oxford

Kempson, G. E., Spivey, C. J., Swanson, S. A. V. & Freeman, M. A. R. (1971) *J. Biomechanics* **4**, 597–609

Larsson, S. & Heinegård, D. (1975) *Protides Biol. Fluids Proc. Colloq.* **22**, 245–249

Lipshitz, H., Etheredge, R., III & Glimcher, M. J. (1976) *Trans. Annu. Meet. Orthop. Res. Soc. 22nd, New Orleans*, 96

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mankin, H. J. & Lipello, L. (1970) *J. Bone Jt. Surg.* **52A**, 424–434

Mankin, H. J. & Zarins-Trasher, A. (1975) *J. Bone Jt. Surg.* **57A**, 76–80

Mathews, M. B. & Glagov, S. (1966) *J. Clin. Invest.* **45**, 1103–1111

Matthews, B. F. (1953) *Br. Med. J.* **2**, 660–661

McDevitt, C. A. & Muir, H. (1976) *J. Bone Jt. Surg.* **58B**, 94–101

McDevitt, C. A., Muir, H. & Pond, M. J. (1973) *Biochem. Soc. Trans.* **1**, 287–289

Oegema, T. R., Hascall, V. C. & Dziewiatkowski, D. D. (1975) *J. Biol. Chem.* **250**, 6151–6159

Rao, C. R. (1965) *Linear Statistical Inference and its Applications*, p. 167, John Wiley and Sons, New York

Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. & Strider, W. (1976) *J. Biol. Chem.* **251**, 6439–6444

Scott, J. E. (1973) in *Current Developments in Rheumatology* (Lövgren, O., Boström, H., Olhagen, B. & Sannerstedt, R., eds.), pp. 10–29, A. Lindgren, Mölndal

Scott, J. E. (1975) *Phil. Trans. R. Soc. London Ser. B* **271**, 235–242

Šimůnek, Z. & Muir, H. (1972) *Biochem. J.* **126**, 515–523

Stegemann, H. & Stalder, K. (1967) *Clin. Chim. Acta* **18**, 267–273

Strider, W., Pal, S., Margolis, R. & Rosenberg, L. (1976) *Trans. Annu. Meet. Orthop. Res. Soc. 22nd, New Orleans*, 35

Thyberg, J., Lohmander, S. & Heinegård, D. (1975) *Biochem. J.* **151**, 157–166

Wasteson, Å. (1971) *J. Chromatogr.* **59**, 87–97