Age-related changes in the chemical composition of bovine articular cartilage

The structure of high-density proteoglycans

Hari G. GARG and David A. SWANN

Departments of Biological Chemistry and Surgery, Harvard Medical School at Shriners Burns Institute and Massachusetts General Hospital, Boston, MA 02114, U.S.A.

(Received 18 June 1980/Accepted 11 September 1980)

Proteoglycans were extracted from the articular cartilage of foetal, calf and adult bovine metacarpal–phalangeal joints with 4M-guanidinium chloride. After extraction, the high-density proteoglycans (PG-I fractions) were prepared by sedimentation in two sequential CsCl-density-gradient procedures [Swann, Powell & Sotman (1979) *J. Biol. Chem.* **254**, 945–954]. The PG-I fractions from foetal, calf and adult tissues accounted for 75%, 52% and 46% respectively of the extracted components. The glucosamine, galactose, *N*-acetylneuraminic acid and protein contents increased with age. The overall amino acid compositions of PG-I fractions were similar. Fractionation of PG-I-fraction samples on a Bio-Gel A-50m column indicated that the molecular weight decreased with age. The PG-I fractions were specifically ^3H -labelled by treatment with galactose oxidase followed by reduction with NaB^3H_4 . The ^3H radioactivity was incorporated into both galactose and galactosamine residues of different carbohydrate side chains. The elution profiles of alkaline borohydride-treated foetal, calf and adult PG-I-fraction samples on a Sepharose 6B column showed that the molecular weights of chondroitin sulphate chains were 13 500, 12 000 and 10 500 in foetal, calf and adult tissues respectively. Fractionation of the alkaline borohydride-treated foetal, calf and adult PG-I-fraction samples and ^3H -labelled calf and adult PG-I-fraction samples on a Bio-Gel P-10 column showed that there was an inverse relationship between the low-molecular-weight *O*-linked oligosaccharides and the higher-molecular-weight sialic acid-containing constituents at different ages. The oligosaccharide components of foetal, calf and adult PG-I-fraction samples represented 79%, 69% and 36% respectively of the total sialic acid content of the proteoglycans. Similarly in the ^3H -labelled calf and adult samples 75% and 30% of the total radioactivity were present in the oligosaccharide components respectively. Digestion with chondroitinase AC-II and infrared analyses showed that the PG-I-fraction F and C samples contained primarily chondroitin 4-sulphate chains whereas PG-I-fraction sample A was 6-sulphated. These studies show that the major proteoglycans (PG-I fractions) in the articular cartilage of foetal, calf and adult animals differ in the content, types and structure of the chondroitin sulphate, keratan sulphate and oligosaccharide constituents. These changes in proteoglycan structure reflect the gross age-related changes in the chemical composition of the tissue.

Age-related changes in the chemical composition of cartilage are well documented (Kaplan & Meyer, 1959; Anderson *et al.*, 1964; Mathews & Glasgow, 1966; Hjertquist & Engfeldt, 1967, and references cited therein; Greiling & Stuhlsatz, 1969; Bjelle *et al.*, 1972; Hjertquist & Lemperg, 1972; Hjertquist & Wasteson, 1972; Lemperg *et al.*, 1974; Mankin &

Thrasher, 1977). The molecular basis of these changes, however, is not clear. Cartilage proteoglycans are complex polydisperse macromolecules consisting of chondroitin sulphate, keratan sulphate (Rosenberg *et al.*, 1975, 1976; Hardingham *et al.*, 1976; Heinegård & Axelsson, 1977; Heinegård, 1977; Hascall & Heinegård, 1979) and oligo-

saccharide side chains covalently linked to a protein core (Thonar & Sweet, 1979). Several studies (Hoffman *et al.*, 1975; Hopwood & Robinson, 1975; Roughley & Mason, 1976; Stanescu *et al.*, 1977; Swann *et al.*, 1979) have also shown that articular cartilage contains different types of proteoglycans and significant quantities of protein and glycoprotein constituents. There is therefore the possibility that the gross chemical changes may reflect alterations in the structure and/or the proportions of the different types of macromolecular components. To obtain more information about the age-related changes in bovine articular cartilage we have compared the quantities of constituents extracted under dissociative conditions and the structure of the high-density proteoglycans (PG-I fractions) from foetal, calf and adult tissues. Preliminary results of these experiments were reported previously (Garg & Swann, 1980; Swann & Garg, 1980).

After completion of the present studies a paper by Sweet *et al.* (1979) reported the properties of calf and cow articular cartilage proteoglycans. They showed that the major proteoglycan component, referred to as PG-I fraction in the present study, decreased in size with age, and that there was a decrease in the size of the chondroitin sulphate-rich region of the proteoglycans and in the number and apparent molecular weight of the chondroitin side chains. They also observed an increased content of keratan sulphate side chains with age, but they did not find similar age-related changes in the sialic acid-containing oligosaccharide side chains.

Methods

Extraction and fractionation

Proteoglycans were extracted from metacarpal-phalangeal joints of foetal, calf and adult cattle feet with guanidinium chloride containing proteinase inhibitors (1 mM-phenylmethanesulphonyl fluoride, 1 mM-*p*-chloromercuribenzoic acid and 10 mM-disodium EDTA) and fractionated by two dissociative density gradients by using the procedure described by Swann *et al.* (1979).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed with 5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate/0.1 M-sodium phosphate buffer, pH 7.2 (Furthmayr & Timpl, 1971).

General analytical methods

Amino acids, hexosamines, neutral sugars, *N*-acetylneuraminic acid, uronic acid and sulphate were measured by previously described methods (Swann *et al.*, 1979). A manual resorcinol method (Jourdan

et al., 1971) was used to determine the sialic acid content of column fractions (Fig. 8). The values shown in Fig. 6 were obtained by using an automated modification (Lohmander *et al.*, 1980) of this method.

^3H radioactivity was determined with a liquid-scintillation counter.

Gel chromatography

Descending-flow columns (100 cm $\times$ 2.5 cm and 21 cm $\times$ 1.5 cm) packed with Bio-Gel A-50m (100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and a 100 cm $\times$ 2.5 cm column packed with Bio-Gel A-1.5m (100–200 mesh) were equilibrated and eluted with 0.5 M-guanidinium chloride at room temperature. The columns were calibrated with Blue Dextran, bovine serum albumin and $^3\text{H}_2\text{O}$ (New England Nuclear, Boston, MA, U.S.A.). The Bio-Gel P-10 (Bio-Rad) columns (150 cm $\times$ 0.6 cm and 115 cm $\times$ 0.9 cm) and the Sepharose 6B (Pharmacia, Piscataway, NJ, U.S.A.) columns (150 cm $\times$ 0.6 cm) were equilibrated and eluted with 0.5 M-sodium acetate buffer, pH 7.0. These columns were calibrated with adult PG-I fraction and glucuronic acid.

Enzymic and chemical treatment of proteoglycans

Galactose oxidase and NaB^3H_4 . Proteoglycan samples (4 mg) were dissolved in 0.1 M-sodium phosphate buffer, pH 6.0 (1 ml), and treated with galactose oxidase (Worthington, Freehold, NJ, U.S.A.; 50 Worthington units/mg of PG-I fraction; Radin *et al.*, 1969) that had been purified by adsorption chromatography (Hatton & Regoezi, 1976). After incubation for 16 h, the pH of the reaction mixture was adjusted to 7.0 with 2 M- Na_2CO_3 , and the oxidized galactose residues were reduced by reaction with NaB^3H_4 (New England Nuclear; specific radioactivity 325 mCi/mmol), 100 μl of solution containing 0.024 nmol of NaB^3H_4 in 1 mM- NaOH . NaB^3H_4 was destroyed by the addition of 60% (v/v) acetic acid, and the samples were then dialysed against water and freeze-dried.

Chondroitinase ABC. Galactose oxidase-treated and ^3H -labelled proteoglycan samples (5 mg) from foetus, calf and adult were treated with chondroitinase ABC (*Proteus vulgaris*; Miles Laboratories, Elkhart, IN, U.S.A.; 1 unit of enzyme/5 mg of proteoglycan in 1 ml of Tris/HCl buffer, pH 7.8; Saito *et al.*, 1968) at 37°C for 24 h. The reaction mixture was then chromatographed on the Bio-Gel A-1.5m column and the fractions were analysed to determine the ^3H radioactivity and the uronic acid content.

Chondroitinase AC-II. PG-I-fraction samples were treated with chondroitinase AC-II (Miles) under the same conditions as used for chondroitinase

ABC described above except that 3 units of activity were used in each test. The digests were chromatographed on Whatman no. 1 paper (Saito *et al.*, 1968) to separate the disaccharide products. After chromatography, the paper was dried, cut into 1 cm strips and extracted with water at 37°C for 20 h. The absorbance of the extracts at 232 nm was then measured.

Alkaline borohydride. PG-I fraction (5 mg) or galactose oxidase-treated and ^3H -labelled PG-I fraction (2 mg) were treated with 10 mM-NaOH/1 M-NaBH₄ by using the procedure described by Carlson (1968). After neutralization with acetic acid, a portion of unlabelled PG-I fraction was applied to the 150 cm $\times$ 0.6 cm Bio-Gel P-10 column and the remainder to the Sepharose 6B column. With galactose oxidase-treated and ^3H -labelled PG-I fraction the total sample was analysed on the 115 cm $\times$ 0.9 cm Bio-Gel P-10 column. The column fractions obtained with PG-I fraction were analysed to determine the content of uronic acid and of sialic acid. The ^3H -labelled PG-I fractions were analysed to determine the presence of ^3H radioactivity and the sialic acid content.

Identification of ^3H -labelled sugar. Galactose oxidase- and NaB $^3\text{H}_4$ -treated PG-I-fraction samples were hydrolysed with 4 M-HCl at 100°C for 4 h and freeze-dried. The hydrolysates were analysed by t.l.c. on silica-gel plates (Brinkman Instruments, Westbury, NY, U.S.A.) with two solvents (A, propan-2-ol/ethyl acetate/water, 3:3:2, by vol.; B, ethyl acetate/pyridine/acetic acid/water, 36:36:7:21, by vol.). Galactose and galactosamine hydrochloride were used as standards. The presence of galactose and galactosamine was detected by using an anisaldehyde spray reagent (conc. H₂SO₄/anisaldehyde/ethanol/acetic acid, 1:1:18:0.2, by vol.; Dunphy *et al.*, 1966). The ^3H -labelled constituents were detected by measuring the radioactivity in 1 cm $\times$ 1 cm portions of silica gel removed from the t.l.c. plates.

Physical methods

Infrared measurements. Thin films of proteoglycan samples were cast on AgCl discs by dissolving 1 mg of the freeze-dried sample in 2 drops of 10 mM-HCl. The films were analysed by using a Perkin-Elmer model 234 spectrophotometer.

Sedimentation velocity. Sedimentation-equilibrium analyses were performed as previously described (Swann *et al.*, 1976).

Viscosity measurements. Viscosity measurements were performed as previously described (Swann & Caulfield, 1975; Swann *et al.*, 1976).

Light-scattering measurements. Molecular weights were kindly determined by Dr. Fred Silver, with the use of a Chromatix KMX-6 light-scattering device as previously described (Silver *et al.*, 1979).

Results and discussion

The initial dissociative density-gradient procedure (G-I) used to fractionate the tissue extracts yielded fractions with high (B), medium (M) and low (T) buoyant densities. The G-I-B fractions were then refractionated in a second gradient (G-II) to obtain a high-buoyant-density fraction (G-II-B) containing most of the extracted proteoglycans (PG-I fraction). The freeze-dried weights of the various gradient fractions obtained from the foetal, calf and adult tissues are shown in Table 1. There was an increase in both the total amounts of constituents extracted and also of high-density components with increasing age. The adult and calf tissues, however, contained almost 3-fold the quantity of low-density constituents present in the foetal tissue, with the result that the relative proportion of G-I-B constituents as a percentage of the total extracted decreased with age. The results obtained previously (Swann *et al.*, 1979) showed that the adult low-density fraction contained small amounts of a proteoglycan-containing fraction (PG-III) that was different from

Table 1. Recovery of non-diffusible freeze-dried constituents in the density-gradient fractions
For details see the text.

Fraction	Foetal		Calf		Adult	
	(mg/g wet wt. of tissue)	(% of total)	(mg/g wet wt. of tissue)	(% of total)	(mg/g wet wt. of tissue)	(% of total)
G-I-B	14.0	80.7	18.75	59.5	22.75	49.0
G-I-M	1.23	7.1	2.12	6.7	7.37	16.0
G-I-T	2.12	12.2	10.62	33.7	16.05	35.0
Total ...	17.35		31.49		46.17	
G-II-B	9.10	93.3	13.12	88.1	17.62	93.0
G-II-M	0.53	5.4	1.38	9.3	1.25	6.0
G-II-T	0.12	1.2	0.39	2.6	0.19	1.0

Fig. 1. Electrophoretic patterns of G-I (T) and G-II (T) proteoglycan fractions on 5% polyacrylamide gels in the presence of sodium dodecyl sulphate and 10% 2-mercaptoethanol

Gels 1, 2, 3, 4, 5, 6 and 7 are F.G-I, F.G-II, C.G-I, C.G-II, A.G-I and A.G-II fractions and bovine serum albumin respectively. The origin is indicated by 1, and the tracking dye by 2. For details see the text.

PG-I fraction but was composed primarily of proteins and glycoproteins. The major component was a glycoprotein with a mobility that was similar to that of the cleaved hyaluronic acid-binding region (Heinegård & Hascall, 1974). This glycoprotein was again observed to be the major component in the adult G-I-T fraction, but it was present only as a trace component in calf G-I-T fraction and was barely detectable in the foetal fraction (Fig. 1). There were also other differences in the types and relative proportions of other peptide constituents in the foetal, calf and adult G-I-T fractions. The foetal sample contained a single peptide with a mobility similar to that of the low-molecular-weight link protein (Caterson & Baker, 1979), whereas the adult sample contained three bands with mobilities similar to those of the link proteins. All samples contained peptides with a greater mobility than the tracking dye.

Analyses of the G-II-B proteoglycan fractions showed that they were not contaminated with protein components. Small quantities of proteins were, however, present in the foetal and adult G-II-T fractions (Fig. 1), confirming previous observations (Swann *et al.*, 1979) that two dissociative gradients

Table 2. Chemical composition of proteoglycans PG-I fractions

For details see the text.

	Foetal	Calf	Adult
Amino acid content (residues/1000 residues)			
Aspartic acid	64	70	65
Threonine	56	53	56
Serine	111	96	109
Glutamic acid	150	153	143
Proline	110	110	121
Glycine	118	133	118
Alanine	72	79	70
Half-cystine	3	3	9
Valine	71	71	69
Methionine	4	1	5
Isoleucine	34	34	30
Leucine	81	81	72
Tyrosine	13	25	25
Phenylalanine	34	28	42
Lysine	27	18	21
Histidine	18	16	15
Arginine	34	29	30
Protein content (% w/w)	7.6	10.8	16.3
Carbohydrate content (% w/w)			
Glucosamine	0.34	0.80	7.5
Galactosamine	12.3	11.9	12.6
Xylose	0.97	1.15	1.17
Mannose	Trace	0.48	0.48
Galactose	2.45	4.40	7.86
Fucose	—	0.55	0.96
N-Acetylneuraminic acid	0.38	0.47	0.51
Uronic acid	16.7	13.1	19.8
Sulphate content (% w/w)	12.4	17.5	13.6
Uronic acid/protein ratio	2.19	1.2	1.2
$10^{-6} \times$ Molecular weight	1.95		1.28

are necessary to remove peptides completely from the high-density proteoglycan fractions.

The chemical compositions of the high-density proteoglycans in the foetal, calf and adult G-II-B fractions (referred to below as F.PG-I, C.PG-I and A.PG-I respectively) are given in Table 2. The protein content increased with age from 7.6% (w/w) in F.PG-I fraction to 10.8% (w/w) in C.PG-I fraction to 16.3% (w/w) in A.PG-I fraction. The amino acid compositions were similar and the major amino acids present were proline, glutamic acid, serine and glycine. The compositions are similar to those reported previously (Rosenberg *et al.*, 1976; Heinegård, 1977; Swann *et al.*, 1979). The glucosamine and galactose contents increased markedly with age, indicating an increase in keratan sulphate, whereas the N-acetylneuraminic acid increased only slightly. The F.PG-I fraction had only a trace of mannose and a lower content of xylose in comparison with C.PG-I- or A.PG-I-fraction samples, which contained similar amounts of these sugars. There was also a marked age-related increase in

Fig. 2. Elution profiles of PG-I fractions on a Bio-Gel A-50m column

The column was equilibrated and eluted with 0.5 M-guanidinium chloride. O, F.PG-I fraction; ●, C.PG-I fraction; □, A.PG-I fraction. For details see the text.

fucose content. These changes are thought to reflect increases in the number and types of keratan sulphate and oligosaccharide components. There were also differences in the uronic acid and sulphate contents. Low uronic acid and high sulphate values were obtained with three different C.PG-I-fraction samples. Light-scattering measurements indicated that the F.PG-I-fraction sample had a molecular weight of 1.95×10^6 compared with a value of 1.28×10^6 for A.PG-I fraction. The latter value is very similar to that obtained previously by sedimentation-equilibrium analyses (Swann *et al.*, 1979). Gel chromatography on a Bio-Gel A-50m column (Fig. 2) showed that there was an age-related decrease in the molecular weight of the PG-I-fraction samples. The K_{av} values of the F.PG-I-, C.PG-I- and A.PG-I-fraction samples were 0.24, 0.37 and 0.39 respectively.

Incorporation of radioactivity into PG-I fraction by treatment with galactose oxidase and NaB³H₄

When the F.PG-I-, C.PG-I- and A.PG-I-fraction samples were treated with NaB³H₄ alone there was no incorporation of radioactivity. After galactose oxidase treatment (Radin *et al.*, 1969), however, ³H was incorporated into equal weights of F.PG-I, C.PG-I and A-PG-I fractions in the proportions 12:6:1. T.l.c. of ³H-labelled F.PG-I and C.PG-I fractions after acid hydrolysis (Fig. 3) showed the presence of two radioactive constituents with mobilities corresponding to those of galactose and galactosamine in the two solvent systems used. The ratio of labelled galactose to galactosamine was 2.6 for ³H-labelled C.PG-I fraction compared with a value of 4.2 for ³H-labelled F.PG-I fraction. These results indicate that the galactose oxidase/NaB³H₄ treatments specifically labelled galactose and galactosamine and that the foetal sample contained a higher proportion of terminal galactose residues.

Fig. 3. Comparison by t.l.c. of acid hydrolysates of ³H-labelled C.PG-I fraction (1) and chondroitinase-treated ³H-labelled F.PG-I fraction (2) (expressed as c.p.m.), and galactose (3) and galactosamine (4) as standards

The compositions of solvents A and B and other details are given in the text.

Fractionation of the ³H-labelled C.PG-I- and A.PG-I-fraction samples on a Bio-Gel A-50m column (Fig. 4) showed that ³H radioactivity was eluted in the same position as the uronic acid-containing constituents, but the K_{av} values (0.57 and 0.65 for the ³H-labelled C.PG-I and A.PG-I fractions respectively) were significantly lower than those obtained for the untreated samples (0.37 and 0.39 respectively).

These results indicate that the molecular weights of these proteoglycans were significantly decreased by the galactose oxidase/NaB³H₄ treatments. When ³H-labelled F.PG-I fraction was compared with ³H-labelled C.PG-I and A.PG-I fractions by chromatography on a Bio-Gel A-15m column the K_{av} values were 0.3, 0.56 and 0.6 respectively, which indicates that the molecular weight of the F.PG-I fraction was decreased to a smaller extent than was the case with the C.PG-I- and A.PG-I-fraction samples. The amino acid compositions of the ³H-labelled F.PG-I-, C.PG-I- and A.PG-I-fraction samples after chromatography on the Bio-Gel A-15 m column are shown in Table 3. Serine, glutamic acid, proline and glycine were again the major amino acids, but several differences are

Fig. 4. Elution profiles of galactose oxidase-treated and ^3H -labelled C.PG-I fraction (b) and A.PG-I fraction (a) on a Bio-Gel A-50 m column

The column was equilibrated and eluted with 0.5 M-guanidinium chloride and the fractions were analysed to determine ^3H radioactivity (●), uronic acid (○) and protein (A_{280}) (□). For details see the text.

Table 3. Amino acid compositions of galactose oxidase-treated and ^3H -labelled foetal, calf and adult proteoglycan PG-I fractions

For details see the text.

Amino acid	Composition (residues/1000 residues)		
	Foetal	Calf	Adult
Aspartic acid	70	65	71
Threonine	63	54	49
Serine	115	127	111
Glutamic acid	154	153	151
Proline	105	140	140
Glycine	152	146	140
Alanine	70	63	93
Half-cystine	2	2	6
Valine	50	48	78
Methionine	6	1	10
Isoleucine	32	35	39
Leucine	85	80	77
Tyrosine	11	12	33
Phenylalanine	30	36	30
Lysine	32	18	25
Histidine	1	11	13
Arginine	21	14	35

observed when these values are compared with the compositions of the untreated proteoglycans (Table 2). The *N*-acetylneuraminic acid content of the PG-I-

fraction samples was not altered by the galactose oxidase and NaB^3H_4 treatments.

Chondroitinase ABC treatment of ^3H -labelled PG-I-fraction samples

After chondroitinase ABC treatment the ^3H -labelled F.PG-I-, C.PG-I- and A.PG-I- fraction samples yielded significantly different profiles for the distribution of ^3H radioactivity when fractionated by gel chromatography (Fig. 5). The foetal sample showed a broad peak that was eluted throughout the column volume, the calf sample had a bimodal pattern and most of the label in the adult sample was eluted at a K_{av} value of 0.19. The F.PG-I-fraction digest also contained a portion of the ^3H radioactivity in a uronic acid-containing peak. The use of proteinase inhibitors did not affect the elution profiles of radioactivity obtained by this enzyme treatment.

Alkaline borohydride treatment

After digestion with alkaline borohydride the F.PG-I-, C.PG-I- and A.PG-I-fraction samples were fractionated by chromatography on Bio-Gel P-10 (Fig. 6) and Sepharose 6B (Fig. 7) columns. With the foetal and calf samples the sialic acid-containing constituents were present as broad peaks with K_{av} values of 0.28 and 0.22 respectively (Fig. 6), and

Fig. 5. Bio-Gel A-1.5m chromatography of ^3H -labelled samples obtained by treatment of galactose oxidase-treated and ^3H -labelled F.PG-I fraction (a), C.PG-I fraction (b) and A.PG-I fraction (c) with chondroitinase ABC

The column was eluted with 0.5M-guanidinium chloride. The uronic acid content (O) and ^3H -radioactivity (●) were determined. For details see the text.

three discrete low-molecular-weight oligosaccharide peaks with K_{av} values of 0.61, 0.71 and 0.80 were obtained. The relative proportion of the low-molecular-weight oligosaccharides decreased with age (Table 4). The amount and size of the broad sialic acid-containing peak increased with age. The uronic acid constituents of digests of F.PG-I and C.PG-I fractions were eluted at the column void volume, whereas with the A.PG-I-fraction sample these constituents were eluted at a K_{av} value of 0.07. Chromatography of the enzyme digests on the Sepharose 6B column gave single uronic acid-containing peaks with K_{av} values of 0.58, 0.60 and 0.65 for the F.PG-I-, C.PG-I- and A.PG-I-fraction samples respectively (Fig. 7). This indicates a decrease in the apparent molecular weight of the chondroitin sulphate chains with age (see Table 4).

When the distributions of ^3H -labelled constituents in ^3H -labelled C.PG-I- and A.PG-I-fraction samples were compared after treatment with alkaline borohydride, very different patterns were obtained (Fig. 8). The calf sample gave sialic acid-containing peaks with K_{av} values of 0.59, 0.71 and 0.84 and ^3H -radioactivity peaks with K_{av} values of 0.50, 0.62 and 0.86. The adult sample again showed the presence of sialic acid distributed throughout the

Fig. 6. Bio-Gel P-10 chromatography of F.PG-I fraction (a), C.PG-I fraction (b) and A.PG-I fraction (c) after treatment with alkaline borohydride

The column was eluted with 0.5M-sodium acetate buffer pH 7.0. The column fractions were analysed to determine the sialic acid content (O) and uronic acid (●). By use of the automated sialic acid method a reading of 300 corresponded to a concentration of $3\text{ }\mu\text{g/ml}$. For details see the text.

column volume, but small quantities of ^3H radioactivity were also present in the low-molecular-weight fractions. The ^3H incorporation increased with decreasing molecular weight for the calf fractions, whereas incorporation was maximal with the adult-sample peak with a K_{av} value of 0.71. The higher incorporation of ^3H into the C.PG-I-fraction sample compared with the A.PG-I fraction is reflected in the much higher radioactivity values in the low-molecular-weight fractions. The A.PG-I-fraction sample contained larger quantities of radioactivity in the higher-molecular-weight constituents, which had a K_{av} of about 0.22 (Fig. 8). There was also an inverse relationship between the sialic acid content of the oligosaccharides and the incorporation of ^3H radioactivity into terminal galactose or galactosamine residues.

Content of chondroitin 4- and 6-sulphates

The proportions of 4- and 6-sulphate in the chondroitin sulphate side chains were determined by paper chromatography after digestion with chondroitinase AC-II (Fig. 9) and by infrared analyses of PG-I fractions. The distributions of sulphated disaccharides showed that F.PG-I- and C.PG-I-

Fig. 7. Sepharose 6B chromatography of F.PG-I fraction (a), C.PG-I fraction (b) and A.PG-I fraction (c) after treatment with alkaline borohydride

The column was eluted with 0.5M-sodium acetate buffer, pH 7.0, and the uronic acid content of the fractions was determined by using a carbazole reaction. For details see the text.

Table 4. Distribution of sialic acid in foetal, calf and adult proteoglycan PG-I fractions and molecular weights of chondroitin sulphate chains present in the different proteoglycans

For details see the text. The percentage distribution of sialic acid in fraction A (high-molecular-weight constituents) and fraction B (low-molecular-weight oligosaccharides) was calculated from the sialic acid concentrations in column fractions 19–40 and 41–56 respectively (Fig. 6).

	Sialic acid (% of total)		Mol.wt. of chondroitin sulphate chains*
	Fraction A	Fraction B	
Foetal	21.2	78.8	13 500
Calf	31.4	68.6	12 000
Adult	64.2	35.8	10 500

* Calculated as described by Wasteson (1971).

fraction samples contained almost exclusively chondroitin 4-sulphate, whereas the A.PG-I fraction contained primarily chondroitin 6-sulphate. Infra-red analyses also showed the characteristic absorption bands at 820 and 775 cm^{-1} (Orr, 1954; Mathews, 1958; Mathews & Inouye, 1961; Lloyd & Dodgson, 1961) for sulphate with an equatorial

Fig. 8. Bio-Gel P-10 chromatography of ^3H -labelled C.PG-I fraction (a) and A.PG-I fraction (c) after treatment with alkaline borohydride

The column was eluted with 0.5M-sodium acetate buffer, pH 7.0. The sialic acid content (O) and ^3H radioactivity (●) were determined. By use of the manual sialic acid method an absorbance of 0.03 at 630nm corresponded to a concentration of 3 $\mu\text{g}/\text{ml}$. For details see the text.

orientation (6-sulphate) in A.PG-I fraction and at 928, 850 and 725 cm^{-1} for sulphate with an axial orientation in the F.PG-I and C.PG-I fractions (4-sulphate).

Sedimentation-velocity and viscosity measurements

When the F.PG-I, C.PG-I and A.PG-I-fraction samples were mixed with hyaluronic acid and aggregate formation was measured by using viscosity and sedimentation analyses, the foetal and calf samples showed similar viscosity increments (Table 5), whereas a slightly larger value was obtained with the adult sample. The $s_{20,w}$ values of the slow-moving and fast-moving boundaries respectively were 14.4S and 18.2S for F.PG-I fraction, 13.7S and 17.8S for C.PG-I fraction and 11.9S and 20.3S for A.PG-I fraction. The data show that the proteoglycans were able to interact with hyaluronic acid and that this was not an age-related property.

General discussion

The results obtained in the present study are similar in several respects to those reported by Sweet *et al.* (1979). There was an age-related decrease in the size of the proteoglycans, in the number and apparent molecular weight of the chondroitin sulphate chains and also an increase in the keratan sulphate content. The present data, however, indicate that, associated with the altered content and structure of the chondroitin sulphate and keratan sulphate side chains, there are also major changes in the structure of the sialic acid-containing oligo-

Fig. 9. Paper chromatography of chondroitinase AC-II-treated F.PG-I fraction (a), C.PG-I fraction (b) and A.PG-I fraction (c)

The chromatogram was run in butan-1-ol/acetic acid/1M-NH₃ (2:3:1, by vol.) and the distribution of the disaccharides was determined by measuring the absorbance at 232nm. For details see the text. Abbreviations: Di-6S, 6-sulphated unsaturated disaccharide; Di-4S, 4-sulphated unsaturated disaccharide; Di-OS, non-sulphated unsaturated disaccharide.

saccharide constituents (Figs. 6 and 7 and Table 4) and the number and distribution of the terminal galactose and/or galactosamine residues (Fig. 4). In addition, it is clear that both the calf and adult PG-I-fraction samples have a significantly different structure from the foetal PG-I fraction, which has a composition more similar to that of the major proteoglycan in nasal-septum cartilage (Hascall & Sajdera, 1969). Associated with the decrease in alkali-labile oligosaccharides there was an increase in the high-molecular-weight sialic acid-containing

Table 5. Interaction of foetal, calf and adult proteoglycan PG-I fractions with hyaluronic acid

The hyaluronic acid and PG-I-fraction solutions contained 25 µg/ml and 2.5 mg/ml respectively. The mixtures were prepared from equal volumes of hyaluronic acid and PG-I-fraction solutions containing 50 µg/ml and 5.0 mg/ml respectively. For details see the text.

Source	Specific viscosity			Increment
	Hyaluronic acid	PG-I fraction	Hyaluronic acid-PG-I fraction	
Foetal	0.01	0.38	1.10	0.71
Calf	0.01	0.40	1.10	0.69
Adult	0.05	0.25	1.18	0.88

constituents, and the changes in the mannose and fucose contents also indicate that there are alterations in the N-linked oligosaccharides. Because pooled tissue samples of somewhat uncertain age were used in these experiments, it is difficult to reduce these analyses to a molecular basis. It is clear, however, that the major proteoglycan in articular cartilage exhibits marked age-related changes in the number and structure of all the carbohydrate side chains.

This work was supported by U.S. Public Health Grants AM 19834 and GM26625. We are grateful to Dr. V. C. Hascall and Dr. Silvana DeLuca for helpful discussion. Alkaline borohydride treatment of unlabelled proteoglycans was done in the Laboratory of Biochemistry, National Institute for Dental Research, Bethesda, MD 20205, U.S.A. We also thank Mr. Stuart Sotman, Mrs. Susan Powell, Ms. Heather Hermann and Ms. Paegan Deering for skilled technical assistance.

References

Anderson, C. E., Ludowieg, J., Harper, H. A. & Engleman, E. P. (1964) *J. Bone Jt. Surg. Am.* Vol. **46**, 1176-1183
Bjelle, A. O., Antonopoulos, C. A., Engfeldt, B. & Hjertquist, S.-O. (1972) *Calcif. Tissue Res.* **8**, 237-246
Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616-626
Caterson, B. & Baker, J. R. (1979) *J. Biol. Chem.* **254**, 2394-2399
Dunphy, P. J., Pennock, J. F. & Whittle, H. J. (1966) *Chem. Ind. (London)* 1549-1550
Furthmayr, H. & Timpl, R. (1971) *Anal. Biochem.* **41**, 330-334
Garg, H. & Swann, D. A. (1980) *Abstr. Natl. Meet. Am. Chem. Soc.* 179th Abstr. 74 (Carbohydr.)
Greiling, H. & Stuhlsatz, H. W. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 449-456
Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127-143

- Hascall, V. C. & Heinegård, D. K. (1979) *Proc. Int. Symp. Glycoconjugates 4th* 341–373
- Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396
- Hatton, C. & Regoeczi, E. (1976) *Biochim. Biophys. Acta* **438**, 339–346
- Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1989
- Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979
- Heinegård, D. & Hascall, V. C. (1974) *J. Biol. Chem.* **249**, 4250–4256
- Hjertquist, S.-O. & Engfeldt, B. (1967) *Acta Pathol. Microbiol. Scand. Suppl.* **187**, 40–41
- Hjertquist, S.-O. & Lemperg, R. (1972) *Calcif. Tissue Res.* **10**, 223–237
- Hjertquist, S.-O. & Wasteson, Å. (1972) *Calcif. Tissue Res.* **10**, 31–37
- Hoffman, P., Mashburn, T. A., Jr., Hsu, D., Trivedi, D. & Diep, J. (1975) *J. Biol. Chem.* **250**, 7251–7256
- Hopwood, J. J. & Robinson, H. C. (1975) *Biochem. J.* **151**, 581–584
- Jourdain, G. W., Dean, L. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 430–435
- Kaplan, D. & Meyer, K. (1959) *Nature (London)* **183**, 1267–1268
- Lemperg, R., Larsson, S. E. & Hjertquist, S.-O. (1974) *Calcif. Tissue Res.* **15**, 237–251
- Lloyd, A. G. & Dodgson, K. S. (1961) *Biochim. Biophys. Acta* **46**, 116–120
- Lohmander, L. S., DeLuca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H. & Heinegård, D. (1980) *J. Biol. Chem.* **255**, 6084–6091
- Mankin, H. J. & Thrasher, A. Z. (1977) *J. Rheumatol.* **4**, 343–350
- Mathews, M. B. (1958) *Nature (London)* **181**, 421
- Mathews, M. B. & Glasgov, S. (1966) *J. Clin. Invest.* **45**, 1103–1111
- Mathews, M. B. & Inouye, M. (1961) *Biochim. Biophys. Acta* **53**, 509–513
- Orr, S. F. D. (1954) *Biochim. Biophys. Acta* **14**, 173–181
- Radin, N. S., Hoff, L., Bradley, R. M. & Brady, R. O. (1969) *Brain Res.* **14**, 497–505
- Rosenberg, L., Hellmann, E. & Kleinschmidt, A. K. (1975) *J. Biol. Chem.* **250**, 1877–1883
- Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. & Strider, W. (1976) *J. Biol. Chem.* **251**, 6439–6444
- Roughley, P. J. & Mason, R. M. (1976) *Biochem. J.* **157**, 357–367
- Saito, H., Yamagata, T. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
- Silver, F. H., Langley, K. H. & Trelstad, R. L. (1979) *Biopolymers* **18**, 2523–2535
- Stanescu, V., Maroteau, P. & Sobczak, E. (1977) *Biochem. J.* **163**, 103–109
- Swann, D. A. & Caulfield, J. B. (1975) *Connect. Tissue Res.* **4**, 31–39
- Swann, D. A. & Garg, H. G. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1638
- Swann, D. A., Powell, S., Broadhurst, J., Sordillo, E. & Sotman, S. (1976) *Biochem. J.* **157**, 503–506
- Swann, D. A., Powell, S. & Sotman, S. (1979) *J. Biol. Chem.* **254**, 945–954
- Sweet, M. B. E., Thonar, E. J.-M. A. & Marsh, J. (1979) *Arch. Biochem. Biophys.* **198**, 439–448
- Thonar, E. J.-M. A. & Sweet, M. B. E. (1979) *Biochim. Biophys. Acta* **584**, 353–357
- Wasteson, Å. (1971) *J. Chromatogr.* **59**, 87–97