Biochem. J. (1971) **121**, 261–270
Printed in Great Britain

Heterogeneity of Protein–Polysaccharides of Porcine Articular Cartilage

THE SEQUENTIAL EXTRACTION OF CHONDROITIN SULPHATE–PROTEINS WITH ISO-OSMOTIC NEUTRAL SODIUM ACETATE

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(*Received* 29 *July* 1970)

Protein–polysaccharides of knee-joint cartilage of 9-month-old pigs were extracted sequentially with neutral iso-osmotic sodium acetate after five repeated homogenizations. One-third of the uronic acid originally present in the tissue was brought into solution, about half being in the first extract. The protein–polysaccharides, which were purified by precipitation with 9-aminoacridine, were heterogeneous in size on gel chromatography. The smallest (retarded by 6% agarose) were the most easily extracted since they were most prevalent in the initial extracts and absent from later ones, whereas the proportion of larger molecules increased progressively in successive extracts. Nevertheless a small proportion of the largest molecules (excluded from Sepharose 2B) was present even in the first extract. None of the protein–polysaccharide preparations contained hydroxyproline, and the analyses of their constituent sugars were the same, although there was a progressive increase in the protein content and in the glucosamine/galactosamine molar ratio of successive extracts. In each preparation this molar ratio was invariably greater in larger than in smaller molecules separated by gel filtration. From galactosamine/pentose molar ratios it appeared that the chondroitin sulphate chains were on average about 29 disaccharide units in length in the protein–polysaccharides of each extract, although gel-chromatography and cetylpyridinium chloride elution profiles showed that a somewhat higher proportion of shorter chondroitin sulphate chains occurred in the larger protein–polysaccharides. In the last extract, where the largest molecules predominated, about half could be reversibly dissociated by urea, whereas this had no effect on the protein–polysaccharides of earlier extracts even though these contained some large molecules.

When cartilage is suspended in water and homogenized at high speeds (Malawista & Schubert, 1958) most of the protein–polysaccharides (as defined by Tsiganos & Muir, 1969*a*) are solubilized, but those extracted vary extensively in size and in chemical composition (Pal, Doganges & Schubert, 1966; Franek & Dunstone, 1967; Rosenberg, Schubert & Sandson, 1967). Whether the heterogeneity observed among the protein–polysaccharides under these conditions is an artifact of the extraction procedure or whether it accurately represents the state of these molecules in the tissue may be questioned, since Sajdera & Hascall (1969) showed that shearing forces during high-speed homogenization lower the average sedimentation coefficient of protein–polysaccharide, and Lucy, Dingle & Fell (1961) found that exposure of embryonic cartilage to water results in the release of lysosomal protease, and proteolytic enzymes active at neutral pH have also been found in extracts of cartilage protein–polysaccharides prepared by high-speed homogenization (Partridge, Whiting & Davis, 1965; Serafini-Fracassini, Peters & Floreani, 1967). However, even when lysosomal breakdown was minimized by extracting cartilage in iso-osmotic neutral solutions and employing much less vigorous homogenization, the protein–polysaccharide extracted remained heterogeneous in size and in chemical composition (Tsiganos & Muir, 1969*a*). Such extraction methods, chosen for their mildness, did not release the bulk of the protein–polysaccharide of the tissue, but they enabled the protein–polysaccharides of smallest size, which are minor constituents, to be demonstrated in extracts of pig laryngeal (Tsiganos & Muir, 1969*a*) and articular (Brandt & Muir, 1969*a*) cartilage, where they were more prevalent in initial than in subsequent extracts, which contained protein–polysaccharides of increasing size.

The present paper is an extension of a previous

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study (Brandt & Muir, 1969*a*), where it was found that different populations of protein–polysaccharides of articular cartilage were extracted selectively by sodium acetate followed by calcium acetate. It describes the results of extracting incompletely disintegrated pig knee-joint cartilage with iso-osmotic sodium acetate until no more protein–polysaccharides were obtained. A preliminary report of some of these studies has appeared (Brandt & Muir, 1969*b*).

MATERIALS AND METHODS

All reagents were of analytical grade, including acetone and ethanol (RR grade: James Burrough Ltd., London S.E.11, U.K.), with the exception of 9-aminoacridine hydrochloride, glucosamine hydrochloride, galactosamine hydrochloride, glucuronolactone, galactose, xylose, cetylpyridinium chloride (A.B. Reciep Pharmaceuticals, Stockholm, Sweden) and acetylacetone, which was redistilled (b.p. 133–134°C). For chemical analysis all samples were dried to constant weight at 80°C *in vacuo* over P_2O_5.

Analytical methods

Determination of hexuronic acid. Hexuronic acid was determined by a modification (Bitter & Muir, 1962) of the method of Dische (1947) with glucuronolactone as a standard.

Determination of hexosamine. Samples were hydrolysed in 8M-HCl (Aristar) *in vacuo* for 3h at 95°C (Swann & Balazs, 1966). Total hexosamine content was determined as described by Muir & Jacobs (1967) by the Elson & Morgan (1933) reaction, by using a modification of the distillation procedure of Cessi & Piliego (1960), with glucosamine hydrochloride, recrystallized to constant optical rotation, as standard. The optical dispersions of aqueous solutions were determined after 8h at 25°C.

Determination of pentose. The anthrone method of Tsiganos & Muir (1966) was used to determine pentose, with xylose as standard. Interference from other sugars was allowed for by adding to the control tubes galactose, glucuronolactone and glucosamine in amounts similar to those in the samples.

Determination of hexose. The method of Trevelyan & Harrison (1952) was used, with galactose as the standard. Interference by uronic acid was allowed for by the addition of glucuronolactone to control tubes in amounts similar to those present in the sample.

Determination of protein. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine serum albumin as a standard.

Determination of hydroxyproline. Samples were hydrolysed in 6M-HCl (Aristar) at 105°C for 24h and the hydrolysates were neutralized by vacuum desiccation over P_2O_5. Hydroxyproline was determined by the method of Kivirriko, Laitinen & Prockop (1967).

Glucosamine/galactosamine molar ratios. Molar ratios of the two hexosamines were determined by using an amino acid analyser as described by Tsiganos & Muir (1969*b*). Samples were hydrolysed in 8M-HCl at 95°C *in vacuo* for 3h (Swann & Balazs, 1966) and then concentrated to dryness by rotary evaporation at 40°C.

Determination of dry weights. Samples of cartilage from five different animals were blotted lightly with a dry gauze, diced and weighed. The weighed samples were placed in acetone for 48h, during which time the acetone was changed twice, and dried to constant weight at 80°C *in vacuo* over P_2O_5.

Analysis of whole tissue. To determine the total amounts of hydroxyproline, uronic acid and hexosamine and the glucosamine/galactosamine ratios in the whole tissue, samples of about 30mg of dried cartilage from each of three animals were digested with papain at 65°C for 24h under toluene in 0.2M-sodium acetate buffer, pH5.7. For each sample of dried cartilage approx. 4mg of crude enzyme (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was used in 3ml of buffer containing 4mg of EDTA (disodium salt) and 1mg of cysteine hydrochloride. To 1ml portions of the digests equal volumes of conc. HCl (Aristar) were added and the solutions heated in sealed tubes for 24h at 106°C. The hydroxyproline content of each tube was determined after evaporation of the hydrolysates to dryness *in vacuo*. To the remainder of the papain digests a slight excess of a warm saturated solution of 9-aminoacridine hydrochloride was added slowly to precipitate the glycosaminoglycans. The glycosaminoglycans were isolated and purified essentially by the same methods as described below for the protein–polysaccharide and then analysed for uronic acid, hexosamine and glucosamine/galactosamine molar ratios.

Extraction of protein–polysaccharides. Normal knee joints from pigs about 9 months old were frozen in solid CO_2 within 30min of death. The joints were subsequently thawed to 4°C, the joint capsules were opened and synovial fluid was wiped from the cartilage surfaces as completely as possible. The cartilage was then shaved with a scalpel from femoral condyles and femoral surfaces of the patellas. Samples (about 0.15g) were removed for the determination of dry weight. The remainder of the tissue was diced in the cold and then immediately frozen in liquid N_2 and pulverized by hammering in a steel die cooled in liquid N_2. The resulting granular tissue was extracted as follows. Portions (15g) of pulverized cartilage were suspended in 100ml of cold 0.15M-sodium acetate buffer, pH6.8, and the suspensions agitated for 10min at low speed in a small MSE homogenizer. The combined homogenates were filtered through two layers of lint and washed three times with 50ml of 0.15M-sodium acetate buffer. The clear filtrate and washings were combined and the protein–polysaccharides immediately isolated with 9-aminoacridine hydrochloride and purified by a second precipitation with 9-aminoacridine (Tsiganos & Muir, 1969*a*), except that the precipitate was collected by centrifugation rather than filtration and Dowex 50 (Na^+ form) was used in place of Zeo-Karb 225 for the conversion of the protein–polysaccharides into their sodium salts. The protein–polysaccharide thus obtained was designated extract 1. The cartilage residue was resuspended in 500ml of fresh sodium acetate buffer and shaken in the cold overnight, after which the suspension was filtered and washed. The protein–polysaccharide in the filtrate, designated extract 1W, was isolated and purified in the same fashion as in extract 1, as were the protein–polysaccharides in each of the subsequent extracts. Four additional homo-

genizations were carried out for 1 h each in 500 ml of sodium acetate buffer and the suspension was filtered and washed as with extract 1. After the second and third homogenizations the residual cartilage was shaken overnight in the cold with 500 ml of fresh sodium acetate buffer. This gave, in all, extracts 1–5 and washings 1W–3W. The second and third homogenizations were performed at low speed in the small homogenizer, and the fourth and fifth at top speed in a larger MSE homogenizer (Ato-Mix). All suspensions were cooled in a bath of solid CO_2/acetone so that the temperature during homogenization did not rise above 4°C.

Histology. Samples of pulverized cartilage suspended in iso-osmotic sodium acetate before the first homogenization and pieces of the residue at various stages of the extraction procedure were wiped free of excess of solvent, rinsed briefly with ethanol, fixed for 24 h in ethanol, embedded in paraffin, sectioned and stained with Toluidine Blue (Pearse, 1968).

Chromatographic methods

Gel chromatography. Gel chromatography of protein–polysaccharides on agarose gels of different pore sizes was performed as follows: 6% (w/v) agarose (a gift from C. P. Tsiganos) was used in two columns (45 cm × 1.8 cm and 47 cm × 0.9 cm); Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in two columns (60 cm × 1.8 cm and 50 cm × 0.9 cm); Sepharose 2B (Pharmacia Fine Chemicals) in a column (55 cm × 2.1 cm). When the three wider columns were used, 5–6 mg samples of protein–polysaccharide in 2.5 ml were applied and the columns eluted with 0.5 M-sodium acetate buffer, pH 6.5, 5 ml fractions being collected at a rate of 15 ml/h. When the two narrower columns were used, 2.5 mg of protein–polysaccharide in 0.5 ml were applied and the columns eluted with the same solvent, 0.9 ml fractions being collected at 2.7 ml/h.

The molecular size of the constituent chondroitin sulphate chains of the protein–polysaccharide fractions was assessed by gel chromatography on Sephadex G-200 (Pharmacia Fine Chemicals) (Wasteson, 1969) after papain digestion. Samples of the protein–polysaccharide (about 8 mg) were suspended in 2 ml of 0.1 M-sodium acetate buffer, pH 5.5, containing 2 mg of EDTA and 0.6 mg of cysteine hydrochloride, to which 3 mg of crude papain (Koch–Light Laboratories) was added. Digestion was carried out under toluene for 24 h at 65°C, after which the glycosaminoglycan was isolated by precipitation with 85% (v/v) ethanol. The precipitated glycosaminoglycan was washed with 85% (v/v) ethanol, absolute ethanol and then with acetone and dried *in vacuo*. Samples (about 6 mg) of glycosaminoglycan were applied to a column (170 cm × 1.1 cm) and eluted with 0.2 M-sodium acetate buffer, pH 6.5. Fractions (3 ml) were collected at a rate of 15 ml/h.

All gel chromatography was performed at 4°C, and to ensure complete dissolution samples were stirred in the eluting solvent overnight in the cold. The uronic acid content of effluent fractions from each column was determined and the amounts of protein–polysaccharide excluded and retarded by the agarose gels were discerned by pooling respective fractions, which were then dialysed against water, concentrated by rotary evaporation and analysed for their uronic acid contents.

To reisolate the protein–polysaccharide in the retarded and excluded fractions the contents of the tubes containing the appropriate fractions were pooled and concentrated by ultrafiltration under reduced pressure in previously heated dialysis tubing (Callanan, Carrol & Mitchell, 1957). The protein–polysaccharides were then precipitated with 85% (v/v) ethanol, and washed with 85% (v/v) ethanol, absolute ethanol, acetone and dried *in vacuo* over P_2O_5.

Gel chromatography after disaggregation by 8 M-urea in 2 M-sodium acetate buffer, pH 6.8. To study whether aggregation affected the size of protein–polysaccharide on gel chromatography, samples of protein–polysaccharide from each extract were dissolved overnight in a solution of 8 M-urea in 2 M-sodium acetate buffer, pH 6.8. Samples (0.5 ml) of this solution, containing about 2.5 mg of protein–polysaccharide, were applied to the smaller Sepharose 4B and 6% agarose columns, which were eluted with 0.5 M-sodium acetate buffer, pH 6.5, and the uronic acid content of each of the fractions of the effluent was determined. The protein–polysaccharide in the retarded and excluded fractions was dialysed against water, concentrated by rotary evaporation when necessary and reisolated by precipitation with 85% (v/v) ethanol to which a few drops of sodium acetate solution (containing 30 g of anhydrous sodium acetate and 15 ml of acetic acid made up to 100 ml with water) were added. The precipitates were washed with 85% ethanol, absolute ethanol and acetone and dried *in vacuo* over P_2O_5, and the glucosamine/galactosamine molar ratio was determined. The remaining urea solutions, which had not been chromatographed, were dialysed for 24 h against several changes of the eluting buffer and the protein–polysaccharide in the dialysis residues precipitated by adding ethanol to 85%. The precipitated protein–polysaccharides were then redissolved in small volumes of 0.5 M-sodium acetate buffer, pH 6.5, and the uronic acid content determined. The volumes were adjusted until all were about equimolar with respect to uronic acid when 0.5 ml of each solution, containing about 2.5 mg of protein–polysaccharide, was chromatographed as before on columns of 6% agarose and Sepharose 4B.

Micro-column fractionation of glycosaminoglycans as cetylpyridinium chloride complexes. Fractionations on micro-columns were performed as described by Antonopoulos, Gardell, Szirmai & De Tyssonsk (1964). The isolated purified protein–polysaccharides (750 μg) were dissolved in 250 μl of 50 mM-sodium phosphate buffer, pH 6.5, containing 5 mM-cysteine and 50 mM-EDTA (disodium salt). Then 5 μl of papain suspension in 0.05 M-sodium acetate containing about 1 i.u. (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; 2× crystallized; 10 i.u./mg of protein) was added, and the digestion carried out at 60°C for 5 h. The digest was then evaporated to dryness in a vacuum desiccator over P_2O_5 and redissolved in exactly 250 μl of 5 mM-Na_2SO_4. Micro-columns (3 mm × 30 mm), packed under slight pressure with Whatman CF 11, cellulose, were equilibrated with 3 ml of 1% cetylpyridinium chloride in 5 mM-Na_2SO_4. Then 50 μl of the redissolved sample (containing approx. 150 μg of glycosaminoglycan) was applied to each of duplicate columns and allowed to soak in completely. Elution was carried out with successive 1 ml volumes of salts of increasing concentration in 0.05% cetylpyridinium chloride, as shown in Fig. 1. The glycosaminoglycans in the eluates

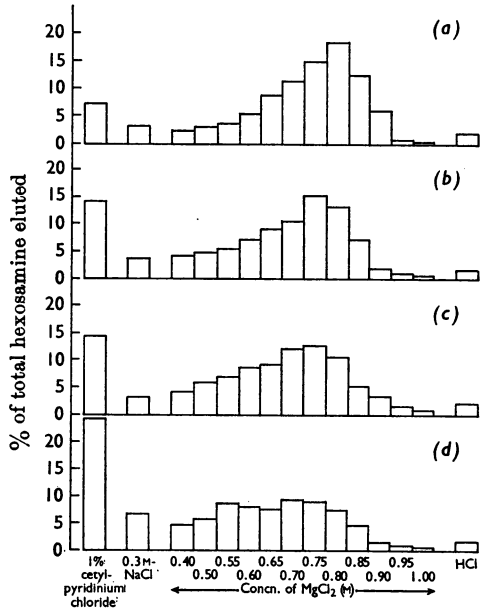

Fig. 1. Solubility profiles on cellulose micro-columns (30mm × 3mm) of cetylpyridinium complexes of glycosaminoglycans obtained by papain digestion of protein–polysaccharides found in sequential sodium acetate extracts. About 150μg of glycosaminoglycan was applied in 50μl of 5mM-Na_2SO_4. The columns were eluted in stepwise fashion with aqueous 1% (w/v) cetylpyridinium chloride followed by increasing concentrations of NaCl and $MgCl_2$ in 0.05% (w/v) cetylpyridinium chloride as shown, and the eluates analysed for their hexosamine contents. (*a*) Fraction of extract 1 retarded on 6% agarose; (*b*) fraction of extract 1 excluded by 6% agarose; (*c*) extract 5; (*d*) fraction of extract 5 excluded by Sepharose 2B.

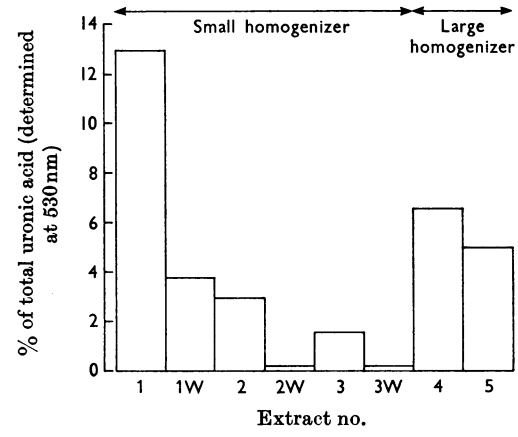

Fig. 2. Relative yields of sequential sodium acetate extracts, expressed as the percentage of the total tissue uronic acid. See the text for details of the sequential extraction.

were determined as hexosamine by the Elson–Morgan reaction, essentially as described by Antonopoulos *et al.* (1964).

RESULTS

General chemical composition. The dry weight of the tissue was 20±0.2% of the wet weight. The total uronic acid and hexosamine contents, derived from analyses of the glycosaminoglycan isolated after complete proteolysis of the tissue, were 2.8±0.3% and 2.8±0.1% respectively, the glucosamine/galactosamine molar ratio being 1:17.5. The tissue hydroxyproline content was 8.0±0.3%.

Gross and histological appearance. When the cartilage had been pulverized in liquid nitrogen and suspended in 0.15M-sodium acetate, the smallest pieces, which were less than 1mm^3 in size, were gelatinous and translucent, whereas the largest, approx. 2–3mm^3 in size, remained grossly indistinguishable from the original cartilage. Both larger and smaller particles showed intense metachromasia of the matrix with Toluidine Blue.

After the series of extractions in sodium acetate the macroscopic appearance of the residue had changed little. The only apparent effect of the larger homogenizer was to decrease somewhat the number of larger pieces of cartilage. Histological examination showed only that some chondrocytes had been dislodged from lacunae, but there was no loss of metachromasia.

Sequential extraction. Protein–polysaccharides in the series of sodium acetate extracts accounted for about 34% of the total uronic acid of the tissue. Significant amounts of uronic acid were not found in the supernatant solutions after precipitation of each extract with aminoacridine, showing that all the protein–polysaccharides were precipitated by this reagent. As shown in Fig. 2, the proportion of tissue uronic acid extracted with sodium acetate during the first homogenization (extract 1), performed at very low speed for only 10min, was much greater than that extracted after the second and third homogenizations, even though these, though also at very low speeds, were carried out for much longer (extracts 2 and 3). Moreover, a point was reached when further treatment of the residue with the smaller homogenizer failed to release further protein–polysaccharide. However, the more powerful homogenizer, used at top speed, yielded some protein–polysaccharide (extracts 4 and 5), although the yields were far less than in extract 1.

Table 1. *Composition of protein–polysaccharides extracted sequentially from pig knee-joint cartilage with 0.15 M-sodium acetate buffer*, pH 6.8

For further details see the text.

Extract no.	Composition (% of dry wt.) Uronic acid	Hexosamine	Hexose	Xylose	Protein	Glucosamine/galactosamine molar ratio
1	28.4	31.0	4.7	0.8	7.1	1:30
1W	28.6	32.2	4.3	0.9	9.9	1:18
2	28.0	31.5	5.0	0.8	9.5	1:12
2W	27.2	30.8	4.7	0.8	10.1	1:12
3	29.0	32.0	4.6	1.0	9.9	1:10
3W	27.3	—	4.6	—	10.6	—
4	28.2	30.7	5.2	0.8	13.2	1:9
5	26.8	31.4	5.1	0.9	12.5	1:7.8

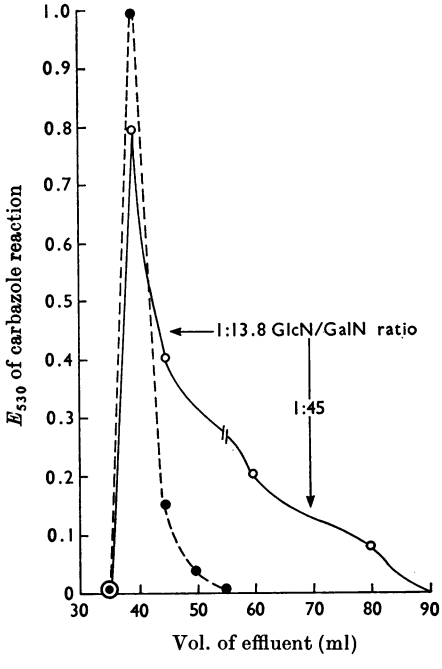

Fig. 3. Elution of protein–polysaccharide from a column (45 cm × 1.8 cm) of 6% agarose with 0.5 M-sodium acetate buffer, pH 6.5, at 4°C. The uronic acid content of each 5 ml fraction was determined. ○, Extract 1; ●, extract 2W. The point at which the excluded and retarded fractions of extract 1 were separated and collected is shown by the break in the solid line. The glucosamine/galactosamine molar ratios were determined in the two fractions and are shown in the figure.

The uronic acid, hexosamine, hexose and pentose contents of the protein–polysaccharide in each extract were, however, similar (Table 1), and none of the extracts contained hydroxyproline. Nevertheless there was a progressive increase in the glucosamine/galactosamine molar ratio and in the apparent protein content of the protein–polysaccharide of sequential extracts (Table 1).

Gel chromatography. In extract 1, the fraction retarded by 6% agarose constituted about 35% of the total protein–polysaccharide (Fig. 3), but the proportion of relatively small protein–polysaccharide diminished successively in extracts 1W, 2 and 2W so that in extract 2W less than 5% of the protein–polysaccharide was retarded on 6% agarose (Fig. 3). In extract 3 and in all subsequent extracts there were no protein–polysaccharides small enough to penetrate 6% agarose and all were eluted in the void volume. The glucosamine/galactosamine molar ratio (Fig. 3) and the relative proportion of protein to uronic acid of protein–polysaccharide in extract 1 excluded from 6% agarose were about three times greater than of the smaller protein–polysaccharide retarded by this gel. The protein–polysaccharides in the fractions of each extract excluded from 6% agarose were isolated and rechromatographed on Sepharose 4B. Fig. 4 shows that the proportion of protein–polysaccharide excluded from this gel increased progressively in successive extracts. It was calculated from the uronic acid contents of the fractions eluted in the void volume that approx. 10% of the protein–polysaccharide in extract 1, 25% of the protein–polysaccharide in extract 3 and 50% of the protein–polysaccharide in extract 5 were large enough to be excluded from Sepharose 4B (Fig. 5).

In each extract the protein–polysaccharide excluded by Sepharose 4B invariably had a higher glucosamine/galactosamine molar ratio than those in each extract retarded by this gel (Fig. 4). The protein–polysaccharide excluded from Sepharose 4B had a higher glucosamine content than that of the same extract excluded from 6% agarose. At the same time the protein–polysaccharides retarded

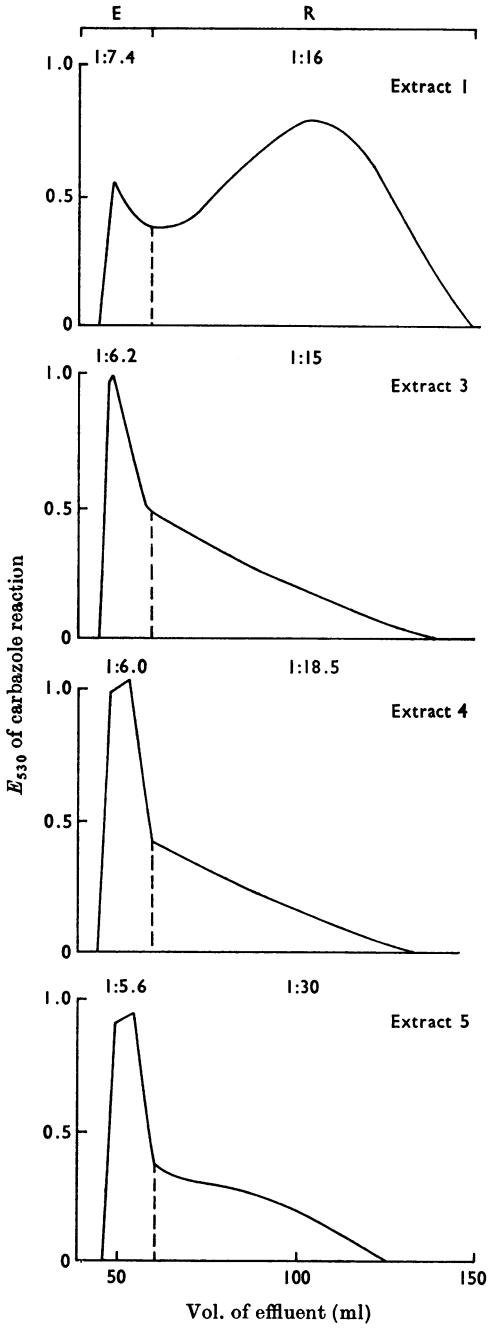

Fig. 4. Elution from a column (60 cm × 1.8 cm) of Sepharose 4B (conditions as in Fig. 3) of protein–polysaccharide in various extracts that were excluded from 6% agarose. The points at which excluded (E) and retarded (R) fractions from Sepharose 4B were separated are shown by the vertical broken lines. Each was isolated and the glucosamine/galactosamine molar ratios were determined. These are shown above each profile.

by Sepharose 4B had a higher proportion of glucosamine than those of smallest size in extract 1 that were retarded by 6% agarose, where the glucosamine/galactosamine ratio was 1:45.

Although the protein–polysaccharides excluded from 6% agarose in successive extracts showed a progressive increase in the glucosamine/galactosamine molar ratio from 1:13.5 in extract 1 to 1:7.8 in extract 5, the protein–polysaccharides excluded from Sepharose 4B were more similar among themselves, and showed a smaller progressive increase in their glucosamine/galactosamine molar ratios (Fig. 4).

Protein–polysaccharides of whole extracts were also chromatographed on Sepharose 2B. The results were analogous to those obtained with gels of lower porosity. Thus increasing proportions of each successive extract were excluded from Sepharose 2B. When the results of chromatography of the successive extracts on Sepharose 4B and 2B were compared, it could be calculated that in each

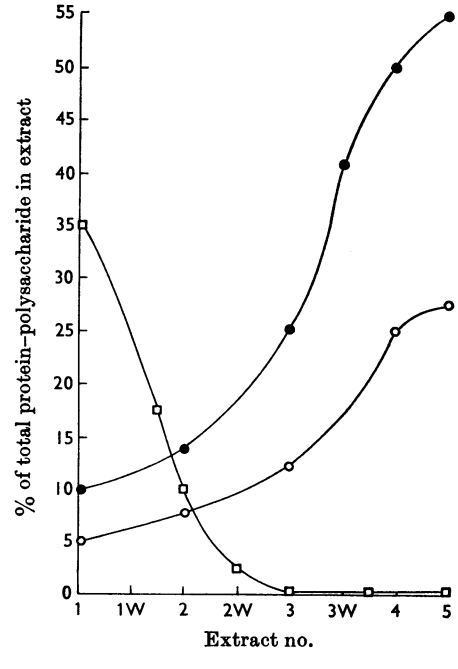

Fig. 5. Proportion of protein–polysaccharide of small, intermediate and large size in sequential sodium acetate extracts, expressed as a percentage of the total protein–polysaccharide in each extract. The proportions were calculated from the uronic acid content of the respective pooled fractions (see the text). □, Small protein–polysaccharide retarded by 6% agarose; ●, protein–polysaccharide of intermediate size retarded by Sepharose 4B; ○, large protein–polysaccharide excluded by Sepharose 4B.

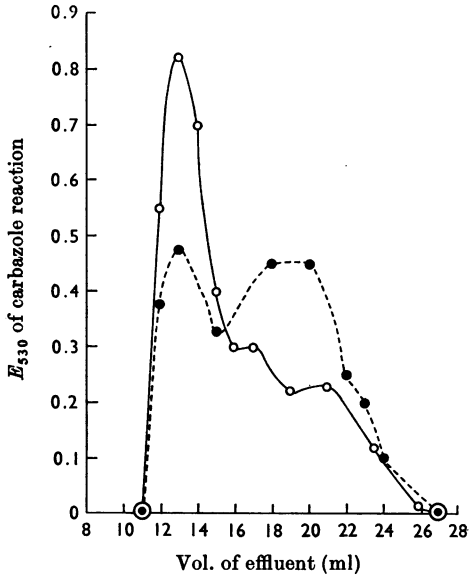

Fig. 6. Comparison of elution profiles of protein–polysaccharides of extract 5 with (●) and without (○) prior treatment with 8M-urea in 2M-sodium acetate buffer, pH 6.8. The protein–polysaccharides were dissolved overnight in the urea solution or in the eluting solvent, and applied to a column (47 cm × 0.9 cm) of Sepharose 4B eluted with 0.5M-sodium acetate buffer, pH 6.5. The uronic acid content of each 0.9 ml fraction was determined.

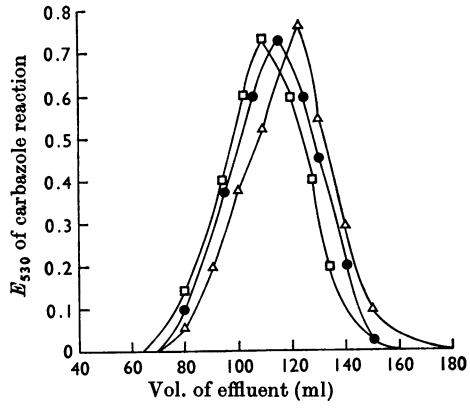

Fig. 7. Gel chromatography of glycosaminoglycan on a column (171 cm × 1.1 cm) of Sephadex G-200 eluted as in Fig. 3. Fractions (3 ml) were collected and the uronic acid contents determined. The glycosaminoglycans were isolated after papain digestion of protein–polysaccharides of various extracts. □, Extract 1; ●, extract 3; △, fraction of extract 5 excluded from Sepharose 2B.

extract about half the protein–polysaccharides excluded from Sepharose 4B were also excluded from Sepharose 2B (Fig. 5), showing that that excluded from Sepharose 4B was not homogeneous with respect to size. The protein–polysaccharides excluded from Sepharose 2B in each extract were reisolated and the glucosamine/galactosamine molar ratios determined. In every extract this ratio, which varied between 1:5 and 1:3.5, was higher than in the protein–polysaccharide of the corresponding fraction excluded from Sepharose 4B.

Effects of 8M-*urea in* 2M-*sodium acetate buffer*, pH 6.8. No change was produced in the elution profiles of extracts 1–3 on chromatography on 6% agarose or Sepharose 4B when protein–polysaccharides in these extracts were dissolved overnight in 8M-urea–2M-sodium acetate buffer, pH 6.8, before chromatography. In sharp contrast, however, the protein–polysaccharides in extracts 4 and 5 were markedly altered by treatment with the urea–sodium acetate solution. As seen in Fig. 6, of the uronic acid of extract 5 that appeared in the void volume when the untreated sample was chromatographed on 6% agarose nearly half penetrated the gel after exposure to the urea–sodium acetate solution. Nonetheless the glucosamine/galactosamine molar ratios of the resulting retarded and excluded fractions of extract 5 were both about 1:7.5, essentially the same as that of the unfractionated protein–polysaccharides of extract 5 (1:7.8). The effects of the urea–sodium acetate solution were fully reversible since after dialysis of the solution against the eluting buffer the elution profile of extract 5 reverted to that of the untreated protein–polysaccharide. Similar, but less pronounced, changes in the elution profile of extract 4 on 6% agarose were seen after exposure of that extract to the urea–sodium acetate solution.

Cetylpyridinium chloride micro-column profiles of glycosaminoglycan chains. Solubility profiles of the cetylpyridinium chloride complexes of glycosaminoglycan chains obtained after proteolysis of the protein–polysaccharides in the various extracts and fractions showed that they had different elution profiles (Fig. 1). A greater proportion of glycosaminoglycans of samples containing more smaller protein–polysaccharides were eluted at somewhat higher salt concentrations than those of samples containing more larger protein–polysaccharides. Most of the glycosaminoglycans, however, were eluted at concentrations of magnesium chloride consistent with their being chondroitin sulphate. Antonopoulos *et al.* (1964) showed that 1% cetylpyridinium chloride elutes keratan sulphate, peptides and protein. Those protein–polysaccharides with the highest protein contents and glucosamine/galactosamine molar ratios (Fig. 1) had the largest proportion of glycosaminoglycans eluted by 1% cetylpyridinium chloride, for example, 18% of glycos-

aminoglycans of protein–polysaccharides in extract 5, where the glucosamine/galactosamine molar ratio was 1:7.8 and 24% of those of protein–polysaccharides in this extract excluded from Sepharose 2B, where the ratio was 1:4.5.

Since the fractionation of glycosaminoglycans on cetylpyridinium chloride–cellulose columns depends on differences in charge or in chain lengths, or both, an attempt was made to relate differences in cetylpyridinium chloride elution profiles to differences in molecular size of chondroitin sulphate chains, by chromatography on Sephadex G-200, as described by Wasteson (1969). The results (Fig. 7) showed that in all fractions the chondroitin sulphate chains were heterogeneous in size. Thus the cetylpyridinium chloride elution profile appeared to be analogous to the profile on Sephadex G-200, in that fractions eluting at higher magnesium chloride concentrations contained more longer chondroitin sulphate chains.

DISCUSSION

As judged by water, collagen and glycosaminoglycan contents, the knee-joint cartilage used in this study resembled that from pigs of the same age analysed previously (Brandt & Muir, 1969*a*), which was subjected to a more limited sequential extraction of its protein–polysaccharides.

In considering the role of protein–polysaccharides in maintaining the structural integrity of articular cartilage it is notable that Hoffman, Mashburn, Meyer & Bray (1967) found no macroscopic change in appearance of the tissue after extracting most of the protein–polysaccharide of bovine nasal cartilage. Curtis & Klein (1963), studying both human and bovine articular cartilage, found that even when all the tissue hexosamine was released by trypsin at 37°C minimal change in the gross appearance of cartilage occurred, although after incubation at 55°C, where collagen would have undergone gelatinization, trypsin produced complete dissolution of the tissue. Hence it is to be expected that when only about one-third of the tissue uronic acid had been extracted there was no change in macroscopic or histological appearance nor loss in metachromasia.

Only about 20% of the tissue uronic acid was extracted after repeated use of the small homogenizer, and almost three-quarters of this was in the first extract. As more vigorous homogenization released some more protein–polysaccharide (extracts 4 and 5) it would appear that these were mechanically entrapped in the collagen network.

The protein–polysaccharides of smallest size (retarded by 6% agarose) were those most easily extracted since the first extract contained much more of them and they were absent from later extracts (Fig. 2). This is consistent with earlier results (Tsiganos & Muir, 1969*b*; Brandt & Muir, 1969*a*), and suggests that the smallest compounds are present *in vivo* and do not result merely from degradation during the extraction procedure. Presumably, because of their relatively small size, they are less easily enmeshed in the collagenous network of cartilage. From the profiles on 6% agarose gel chromatography it was calculated that the smallest protein–polysaccharide accounted for approx. 5% of the total tissue uronic acid.

Conversely the proportion of larger molecules increased progressively in later extracts, although a small proportion of the largest molecules, excluded from Sepharose 2B, was present even in the first extract (Fig. 5). The incomplete sequential extraction procedure used here thus selected molecules approximately according to size, showing that cartilage contains a varied population of protein–polysaccharides of different sizes that may not be distributed uniformly in the tissue.

The glucosamine/galactosamine molar ratio was invariably greater in the larger than in the smaller protein–polysaccharides in every extract. The glucosamine in the purified extracts is attributable to keratan sulphate, since protein–polysaccharides isolated by the procedures used here are free of glycoprotein and hyaluronic acid (Tsiganos & Muir, 1969*b*). Further, in the present study the hexosamine eluted from the cetylpyridinium chloride–cellulose micro-columns with 0.3 M-sodium chloride, which could represent either hyaluronic acid or under-sulphated or depolymerized chondroitin sulphate, or both, did not constitute more than 6% of any extract, and was usually less than 4%, of the total hexosamine eluted. It is thus unlikely that significant amounts of hyaluronic acid were present. The parallel increases in keratan sulphate and protein content and size of protein–polysaccharides noted in this study agree with previous findings on laryngeal (Tsiganos & Muir, 1969*a*,*b*) and articular (Brandt & Muir, 1969*a*) cartilage of pigs also aged about 9 months, but since the total protein and keratan sulphate contents of the various protein–polysaccharides were not large the other analytical values, including uronic acid, did not vary very much (Table 1). Similar results were obtained with immature cartilage of pigs aged 5 weeks (Brandt & Muir, 1969*a*), although the keratan sulphate content was lower than in molecules of comparable size in adult cartilage and there was much less of the protein–polysaccharides of smallest sizes.

If it is assumed that all chondroitin sulphate chains are linked to protein through xylose residues, as in protein–polysaccharides from bovine nasal septum (Rodén & Armand, 1966), the galactosamine/pentose molar ratio will provide an estimate of the average size of chondroitin sulphate chains. This was calculated to be about 29 disaccha-

ride units in each of the extracts. Thus the differences in molecular size of the protein–polysaccharides is unlikely to be due simply to differences in the average length of their chondroitin sulphate chains. Moreover, the small differences that were revealed by gel chromatography and cetylpyridinium chloride elution profiles showed, on the contrary, that larger protein–polysaccharides had more shorter chondroitin sulphate chains. Although it has been suggested that the amounts of chondroitin sulphate attached to core protein may not be the same in all protein–polysaccharides (Hoffman *et al.* 1967), it appears unlikely that the increase in size of protein–polysaccharide can be explained simply by more chondroitin sulphate chains (which are somewhat shorter) being attached to the core protein, particularly since large and small protein–polysaccharides had essentially the same uronic acid content. Tsiganos & Muir (1969*b*) have concluded from comparable analyses of protein–polysaccharides from pig laryngeal cartilage that the increase in size could not be explained merely by an increase in the number of chondroitin sulphate chains attached to one kind of core protein in protein–polysaccharides of all sizes. The concomitant increase of protein and keratan sulphate content with size (as shown here also) and the differences in amino acid composition, *N*-terminal amino acid residues (Tsiganos & Muir, 1969*b*) and antigenic determinants between the smallest and the largest protein–polysaccharides (Tsiganos & Muir, 1969*b*; Brandt, Tsiganos & Muir, 1970) together suggest that cartilage contains a variety of protein–polysaccharides whose core proteins are different and to which differing proportions of chondroitin and keratan sulphate are attached. The significance of this molecular heterogeneity is not known, but there is a somewhat different topographical distribution of keratan sulphate and of chondroitin sulphate with depth from the articular surface of normal human femoral condyles (Maroudas, Muir & Wingham, 1969), which may reflect a difference in the distribution of protein–polysaccharides of different size. Since keratan sulphate contributed relatively more to the compressive stiffness of cartilage of the human femoral head than chondroitin sulphate (Kempson, Muir, Swanson & Freeman, 1970), it is possible that protein–polysaccharides of higher keratan sulphate content, because they are larger than those containing less keratan sulphate, contribute disproportionately more to the physical properties of cartilage.

At least half the protein–polysaccharides in extracts 4 and 5, where the largest molecules predominated, could be reversibly dissociated by 8M-urea in 2M-sodium acetate, after which they became small enough to penetrate 6% agarose. Since the glucosamine/galactosamine molar ratio did not change, the dissociation did not appear to be the same phenomenon as the dissociation of a specific 'link-glycoprotein' rich in glucosamine that may be dissociated in 4M-guanidinium chloride and separated from the protein–polysaccharide of bovine nasal septum by caesium chloride-density-gradient centrifugation (Hascall & Sajdera, 1969).

Not all protein–polysaccharides of large size were affected by urea, however, since 8M-urea did not affect the elution behaviour of protein–polysaccharides of earlier extracts, even though these contained some that were large enough to be excluded by Sepharose 2B. It is notable that 7M-urea and even 4M-guanidinium chloride had no observable effect on the gel-chromatographic behaviour of protein–polysaccharides extracted from pig laryngeal cartilage by sodium acetate after brief low-speed homogenization (Tsiganos & Muir, 1969*a*).

Mr W. Everett, of T. Walls and Sons Ltd., London, U.K., generously supplied the tissue used. We are grateful to Mr D. K. Heinegård for his valuable suggestions on the micro-column fractionations and to Mr R. Ewins for his excellent technical assistance. K. B. is the recipient of a Research Fellowship from the National Cystic Fibrosis Research Foundation. The Arthritis and Rheumatism Council also provided support for this work.

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