Biochem. J. (1971) **123**, 747–755
Printed in Great Britain

Heterogeneity of Protein–Polysaccharides of Porcine Articular Cartilage

THE CHONDROITIN SULPHATE PROTEINS ASSOCIATED WITH COLLAGEN

BY K. D. BRANDT* AND HELEN MUIR
The Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W.6, U.K.

(*Received* 22 *February* 1971)

Pig articular cartilage, from which protein–polysaccharides soluble in iso-osmotic sodium acetate had been removed, was extracted in three further stages with 8M-urea in 2 M-sodium acetate and with tris–HCl buffer after bacterial collagenase digestion, followed by the same urea–sodium acetate solution, thus leaving only 2% of the original uronic acid in the tissue. The histological appearance of the cartilage was unaltered until after collagenase digestion. The collagenase used did not affect the viscosity or molecular size of a protein–polysaccharide preparation obtained previously. The protein–polysaccharides in each extract differed in size, amino acid composition and protein content, but protein and keratan sulphate contents were not related to hydrodynamic size, in contrast with protein–polysaccharides extracted previously before collagenase digestion. Hydroxyproline could not be removed from those obtained by the first urea–sodium acetate extraction until degraded by heat. The galactosamine/pentose molar ratio agreed closely with the galactosamine/serine molar ratio that was destroyed on treatment with 0.5 M-sodium hydroxide, showing that chondroitin sulphate was attached only to serine residues. From these molar ratios the chondroitin sulphate chains were calculated to be of the same average length in protein–polysaccharides in all three extracts although somewhat shorter than in protein–polysaccharides extracted previously. Some threonine residues were also destroyed on alkali treatment suggesting that keratan sulphate may be attached to threonine. These findings together with previous results show that differences in size, composition and physical state extend to all the protein–polysaccharides in cartilage.

In an earlier paper (Brandt & Muir, 1971) the sequential extraction of protein–polysaccharides from pig knee-joint cartilage by repeated homogenization of the tissue in iso-osmotic neutral sodium acetate was described. The protein–polysaccharides thus extracted accounted for 34% of the total tissue uronic acid and consisted of a family of molecules whose size and chemical composition could be related to the ease with which they were extracted from the tissue.

This paper is concerned with the remaining protein–polysaccharides that required more drastic treatment of the cartilage residue to bring them into solution by a sequential procedure. Molecular heterogeneity, which was an overriding feature of the protein–polysaccharides extracted by sodium acetate, also extended to those more firmly bound to the cartilage residue, their molecular parameters differing in each extract.

* Present address: Arthritis and Connective Tissue Disease Section, University Hospital, Boston, Mass., U.S.A.

MATERIALS AND METHODS

All reagents were of analytical grade, except as stated previously (Brandt & Muir, 1971). All samples were dried to constant weight at 80°C *in vacuo* for chemical analysis.

Analytical methods

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

Determination of hexosamine. Hexosamine was determined by a modification (Bitter, 1964) of the procedure of Cessi & Piliego (1960) as described by Brandt & Muir (1971).

Determination of pentose. The method of Tsiganos & Muir (1966) was used to determine pentose, with xylose as standard. Interference from other sugars was allowed for as previously described (Brandt & Muir, 1971).

Determination of hexose. Total hexose was determined by the method of Trevelyan & Harrison (1952), with galactose as standard. Glucuronolactone was added to control tubes in amounts similar to those present in the samples to account for interference by uronic acid.

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

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

Glucosamine/galactosamine molar ratios. Molar ratios of the hexosamines were determined with the use of the 15cm column of the Locarte amino acid analyser, the procedure and the hydrolysis conditions being as described by Brandt & Muir (1971).

Determination of amino acid contents. Samples of protein–polysaccharides (about 10mg) were hydrolysed in sealed tubes under N_2 in 20ml of 6M-HCl (Aristar) for 24h at 105°C, after which the hydrolysates were filtered through no. 4 sintered-glass funnels, which were washed several times with deionized water. The filtrate and washings were combined, evaporated to dryness by rotary evaporation at 40°C and removal of acid was completed by repeated evaporation after addition of further amounts of water. The dried hydrolysates were dissolved in 1ml of starting buffer (0.2M-sodium citrate, pH3.25) and 0.25ml samples were used for analysis on a Locarte amino acid analyser. Elution at pH3.25 was performed for 140min at 52°C followed by 0.2M-sodium citrate buffer, pH4.25, for 220min at 65°C, and finally with 1M-sodium citrate buffer, pH6.65, for 280min at 65°C. The Beckman standard mixture of amino acids was used to determine the individual amino acids by the method of Spackman, Stein & Moore (1958), 0.25μmol of norleucine being included in each analysis as an internal standard.

Alkaline β-carbonyl elimination. Weighed samples (approx. 8mg) of the various extracts were dissolved in 4ml of 0.5M-NaOH and kept at 20°C in sealed tubes under N_2 (Anderson, Hoffman & Meyer, 1965) for 24h, after which 4ml of deionized water and 8ml of conc. HCl (Aristar) were added in an ice bath. Hydrolysis and analysis for amino acids was then carried out as described above.

Chromatographic methods

Gel chromatography. For this 6% agarose (a gift from C. P. Tsiganos) was used in columns 45cm × 1.8cm and 47cm × 0.9cm, and Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in columns 60cm × 1.8cm and 50cm × 0.9cm. Samples of 5–6mg in 2.5ml of 0.5M-sodium acetate, pH6.5, were applied when the larger columns were used and samples of 2.5mg in 0.5ml when the narrower columns were used, the samples being dissolved by stirring at 4°C overnight. The columns were eluted with the sodium acetate solution, fractions of 5ml being collected from the larger columns and 0.9ml from the smaller.

The uronic acid content of the effluent fractions was determined and the amounts of protein–polysaccharides that were excluded and retarded by the gels were determined by pooling respective fractions, which were then dialysed against water, concentrated by rotary evaporation and analysed for their uronic acid contents. The protein–polysaccharides that were retarded and excluded were re-isolated as described previously (Brandt & Muir, 1971).

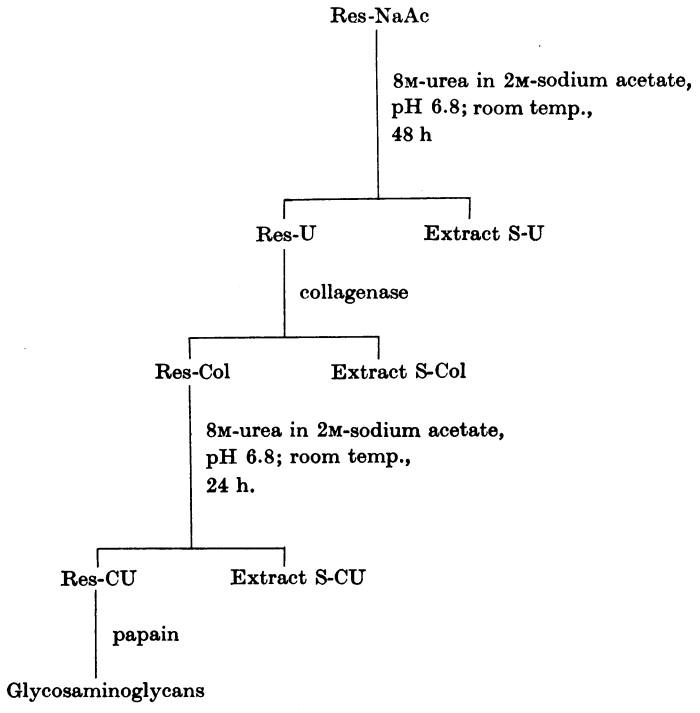
Gel chromatography of protein–polysaccharides after solution in urea–sodium acetate. The protein–polysaccharides in three successive extracts, namely urea extracts of cartilage (S-U), extracts of cartilage after digestion with collagenase (S-Col) and urea extracts of the remaining collagenase-digested cartilage (S-CU), were dissolved overnight in 2M-sodium acetate containing 8M-urea to ascertain whether they could be disaggregated in the same way as the protein–polysaccharides in the final two sodium acetate extracts described previously (Brandt & Muir, 1971). Samples (0.5ml; containing approx. 2.5mg of protein–polysaccharide) were chromatographed on the smaller 6%-agarose and Sepharose 4B columns, which were eluted with 0.5M-sodium acetate, pH6.5, and the uronic acid content of each of the effluent fractions was determined. As controls, samples of each extract were dialysed against the eluting buffer before chromatography (Brandt & Muir, 1971).

Extraction of protein–polysaccharides

As previously reported (Brandt & Muir, 1971) about 34% of the tissue uronic acid had already been extracted. The remaining protein–polysaccharides were then extracted sequentially as follows (Scheme 1).

Urea extract (S-U). The cartilage residue remaining after the final extraction with sodium acetate (Res-NaAc) was suspended in 300ml of 8M-urea in 2M-sodium acetate, pH6.8, and stirred for 48h at room temperature. The suspension was centrifuged at 1400*g* for 30min at 4°C, after which the cartilage residue was washed three times with 50ml portions of the urea solution. The supernatant and washings were pooled and dialysed in previously heated Visking tubing (Callanan, Carrol & Mitchell, 1957) for 48h against running tap water followed by distilled water. The contents and washings of the dialysis sacs were combined and concentrated to approx. 100ml by rotary evaporation at 40°C and the protein–polysaccharides isolated and purified by two precipitations with 9-aminoacridine hydrochloride, after which they were precipitated with ethanol, washed and dried as described by Brandt & Muir (1971).

As extract S-U contained appreciable amounts of hydroxyproline, attempts were made to dissociate the protein–polysaccharides from the collagen, as follows. Samples (3mg/ml) were dissolved overnight by stirring at 4°C in 2M-sodium acetate containing 8M-urea, after which 2 vol. of a saturated solution of 9-aminoacridine hydrochloride was added and the sample was left to stand overnight. After dialysis against several changes of half-saturated aminoacridine, the precipitate formed in the dialysis sac was collected and washed. The protein–polysaccharides were converted into their sodium salts (Brandt & Muir, 1971) and the solution was freeze-dried. The protein–polysaccharides dissolved in a small volume of water were re-isolated and purified by two further precipitations with 9-aminoacridine. In a parallel experiment, another sample of extract S-U (3mg/ml) was dissolved in 0.5M-sodium acetate, pH6.8, and then heated to 60°C for 30min, after which the protein–polysaccharides were re-isolated as described above. In a third experiment, the remainder of extract S-U was dissolved in the 8M-urea–sodium acetate solution. After heating at 80°C for 3h the solution was dialysed against 0.5M-sodium



Scheme 1. Flow diagram of the sequential extraction of protein–polysaccharides from cartilage residue, Res-NaAc, from which 34% of the total uronic acid originally present had already been extracted as described by Brandt & Muir (1971).

acetate, pH6.8, and the protein–polysaccharides were again re-isolated as described above. After conversion into their sodium salts the protein–polysaccharides from each of the three modified S-U preparations were analysed for uronic acid, protein and hydroxyproline, and chromatographed on 6% agarose and Sepharose 4B.

Extract S-Col. After removal of a small sample of the cartilage residue (Res-U, Scheme 1) for histological study the remainder was suspended in 200ml of 0.05M-tris–HCl buffer, pH7.6, containing 1mM-$CaCl_2$ (Rosenbloom, Blumenkrantz & Prockop, 1968), and purified bacterial collagenase was added (50μg/ml) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; code CLSPA; 280 Worthington Units/mg). The digestion was carried out under toluene for 15h at 37°C, after which the cartilage suspension was centrifuged at 1400*g* for 30min at 4°C. The supernatant was decanted and the undigested cartilage washed three times with 50ml portions of the tris–HCl buffer. The supernatant and washings were combined and the protein–polysaccharides precipitated with 9-aminoacridine and then with 85% ethanol, washed and dried in the same manner as extract S-U.

Extract S-CU. Some representative pieces of the cartilage residue from collagenase digestion (Res-Col; Scheme 1) were removed for histological examination and the remainder stirred at room temperature in 200ml of 8M-urea in 2M-sodium acetate, pH6.8, for 24h, during which much of the residue was solubilized. The small amount of insoluble material that remained was separated by centrifugation and washed three times with 50ml portions of 0.15M-sodium acetate, pH6.8. The supernatant and washings were combined and the protein–polysaccharides isolated as above.

The two peaks obtained when extract S-CU was chromatographed on Sepharose 4B were isolated by pooling appropriate fractions and analysed for hydroxyproline. After concentration by rotary evaporation, the protein–polysaccharides in each peak were precipitated with 85% ethanol, washed and dried (Brandt & Muir, 1971). Both fractions were then dissolved in 0.05M-tris–HCl buffer, pH7.6, and redigested with collagenase (50μg/ml) as described by Rosenbloom *et al.* (1968), after which the protein–polysaccharides from each of the peaks were re-isolated with 9-aminoacridine hydrochloride. After conversion of the protein–polysaccharides into their sodium salts (Brandt & Muir, 1971) samples were taken for hydroxyproline analysis and the remainder diluted with 0.5M-sodium acetate, pH6.5, to concentrations of uronic acid approximately the same as that in the original extract. Each peak was then chromatographed separately on Sepharose 4B and the uronic acid content of the effluent fractions determined.

Isolation of residual glycosaminoglycans. Again, a few pieces of the insoluble residue (Res-CU; Scheme 1) were removed for histological examination and the remainder suspended in 30ml of 0.2M-sodium acetate, pH5.5,

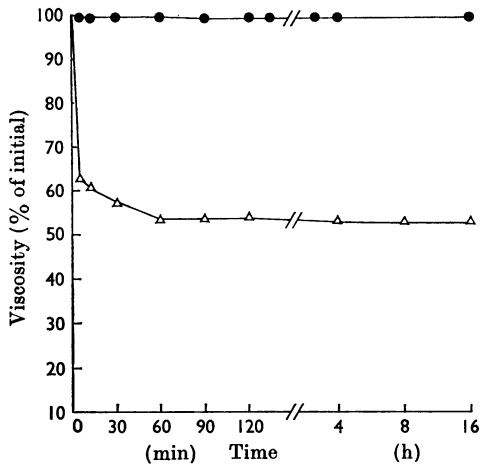


Fig. 1. Effects of purified collagenase and of activated papain at 37°C on the relative viscosity of protein–polysaccharides uncontaminated with any collagen. The percentage of the initial relative viscosity was calculated from the formula $x/y \times 100$, where x is the flow time of the protein–polysaccharide solution after addition of enzyme, and y the flow time of the untreated protein–polysaccharide solution (0.5%) in the first sodium acetate extract (Brandt & Muir, 1971) to which was added collagenase (50 μg/ml) in 0.5 M-tris–HCl buffer, pH 7.6, containing 1 mM-$CaCl_2$ (●), and a solution of same protein–polysaccharide preparation (0.4%) to which was added crude activated papain (Kimmel & Smith, 1954) in 0.02 M-sodium acetate buffer, pH 5.5 (△).

containing 5 mM-EDTA (disodium salt) and 2 mM-cysteine hydrochloride and digested with papain (8 mg/ml) (Kimmel & Smith, 1954) for 24 h under toluene at 60°C. The small amount of undigested material was removed by centrifugation and washed with acetate buffer. Supernatant and washings were combined and the glycosaminoglycans (Scheme 1) precipitated twice with 9-aminoacridine hydrochloride and once with 85% ethanol, washed and dried as with the protein–polysaccharides in each extract.

Confirmation of the absence of non-specific enzymes from the collagenase preparation. Since the collagenase preparation might be contaminated with enzymes that could degrade protein–polysaccharides in tissues and extracts, the effect of collagenase on a preparation of protein–polysaccharides was examined by two methods as follows. (*a*) Protein–polysaccharides obtained from the first sodium acetate extraction of cartilage which were free of collagen and whose behaviour on gel chromatography was known (Brandt & Muir, 1971) were dissolved (5 mg/ml) in tris–HCl buffer (Rosenbloom *et al.* 1968) and collagenase (50 μg/ml) was added. After incubation at 37°C for 15 h under toluene a sample containing approx. 2.5 mg of protein–polysaccharide was applied to the small 6% agarose column and eluted with 0.5 M-sodium acetate, pH 6.5. The control consisted of a sample in the tris–HCl buffer to which no collagenase was added which was incubated for 15 h under toluene and a similar amount applied to the 6% agarose column and eluted in the same way. The uronic acid contents of the eluates of both chromatograms were determined. (*b*) A solution (about 0.5%, w/v) of protein–polysaccharides of the same sodium acetate extract in the tris buffer was stirred overnight at 4°C and then filtered through no. 4 sintered-glass funnels. Samples were placed in Ostwald viscometers, equilibrated at 37 ± 0.05°C, and flow times measured at 6, 12, 30, 60, 90 and 120 min, and then at intervals of 4 h, as shown in Fig. 1. The flow times of samples to which collagenase was added to give a final concentration of 50 μg/ml were determined at the same time-intervals. The decrease in relative viscosity of a 0.4% (w/v) solution of the protein–polysaccharides to which activated papain (10 μg/ml) had been added was also determined (Muir, 1958).

Histology. Representative specimens of cartilage taken at different stages in the extraction sequence were fixed in ethanol for 24 h, embedded in paraffin, cut and stained with Toluidine Blue or van Gieson stain (Pearse, 1968).

RESULTS

The yields, expressed as a percentage of total uronic acid originally present, of the extracts S-U, S-Col, and S-CU and of the glycosaminoglycans released by proteolytic digestion of the final residue, are shown in Fig. 2. Protein–polysaccharides and glycosaminoglycans were completely precipitated by 9-aminoacridine since the supernatants contained virtually no uronic acid. No material containing uronic acid passed through the heated dialysis tubing. Between 9% and 14% of the total tissue uronic acid was recovered in each of the three extracts, the glycosaminoglycans isolated from the final residue (Res-CU) accounting for only about 2% of the total uronic acid. Thus, the three extracts together accounted for the same amount of uronic acid as the protein–polysaccharides in all the previous sodium acetate extracts (Brandt & Muir, 1971).

Histology. The histological appearance of the cartilage was unchanged after treatment with 8 M-urea for 2 days, and neither metachromasia nor van Gieson staining was diminished when compared with normal unextracted cartilage. After collagenase digestion, however, the more peripheral regions of the pieces of cartilage (S-Col-Res; Scheme 1) no longer took up the van Gieson stain, where metachromasia was also decreased. When the cartilage was subsequently extracted with 8 M-urea nearly all metachromasia was lost even though the gross appearance of the cartilage persisted, but after the final proteolytic digestion only a small amount of amorphous material was left, which did not take up the van Gieson stain and lacked metachromasia.

Extract S-U. In contrast with all the earlier extracts (Brandt & Muir, 1971), extract S-U con-

tained much more protein, of which almost half was collagen, since there was 2.15% of hydroxyproline (Table 1). The uronic acid and the protein were wholly excluded from 6% agarose and about half of each was retarded by Sepharose 4B when protein–polysaccharides were dissolved in either 0.5M-sodium acetate, pH 6.8, or in 8M-urea solution before chromatography.

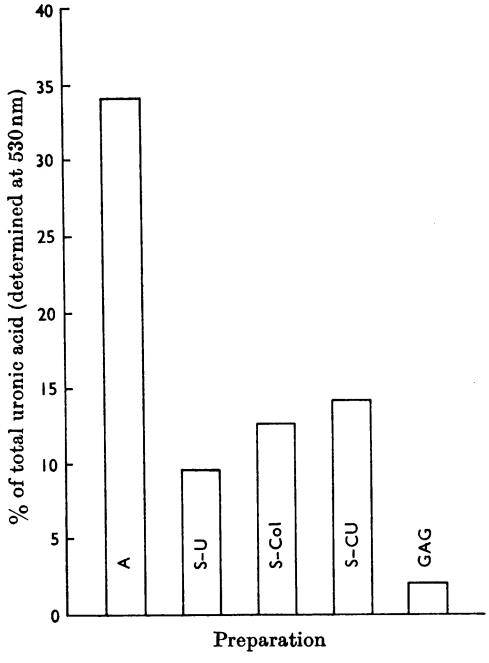


Fig. 2. Yields of protein–polysaccharides in sequential extracts of pig knee-joint cartilage. Protein–polysaccharide uronic acid is expressed as percentage of total uronic acid originally present in the tissue. GAG is the uronic acid due to glycosaminoglycans obtained after the final proteolysis of the tissue. A represents the combined yields of the sodium acetate extractions previously carried out (Brandt & Muir, 1971).

Attempts to dissociate the hydroxyproline and the uronic acid moieties of extract S-U by treatment with urea or by heating at 60°C to denature the collagen were unsuccessful. Although the uronic acid was recovered quantitatively in the re-isolated protein–polysaccharides, 20–40% of the hydroxyproline remained and there was no change in the elution profiles of the protein–polysaccharides. On the other hand, after heating at 80°C for 3h in 8M-urea containing 2M-sodium acetate, pH 6.8, all the uronic acid was recovered and less than 0.1% of the hydroxyproline remained, but the protein–polysaccharides appeared to be extensively degraded, since protein and hexose were largely dissociated from the uronic acid on chromatography on 6% agarose. These protein–polysaccharides were therefore discarded.

Extract S-Col. The protein–polysaccharides that were brought into solution on digestion with collagenase contained 9.2% protein and only traces of hydroxyproline. They differed from those in other extracts because although they were excluded from 6% agarose, they were all retarded by Sepharose 4B and appeared to be rather uniform in size (Fig. 3*a*). Treatment with 8M-urea before gel chromatography did not change the elution profile. The glucosamine/galactosamine molar ratio was 1:11.2, which was similar to that of the protein–polysaccharides in the subsequent extract and of the residual glycosaminoglycans (Table 1).

The amino acid composition of extract S-Col given in Table 2 shows that the protein differed from that of the protein–polysaccharides in the subsequent extract (S-CU) and from those in the fourth sodium acetate extracts of the same cartilage.

Table 1. *Composition of protein–polysaccharides extracted sequentially from pig knee-joint cartilage by the procedures outlined in Scheme* 1 *and of the glycosaminoglycans obtained after the final proteolysis of the tissue*

For further details see the text.

Extract	Composition (% of dry wt.)						Glucosamine/galactosamine molar ratio
	Uronic acid	Hexosamine	Hexose	Xylose	Protein	Hydroxyproline	
5th sodium acetate*	26.8	31.4	5.1	0.9	12.5	—	1:7.8
S-U	20.0	—	—	—	35.3	2.15	1:7.0
S-Col	28.1	29.3	5.8	0.98	9.2	trace	1:11.2
S-CU	25.6	27.1	4.3	0.95	16.7	0.30	1:11.3
Glycosamino-glycans	32.5	34.3	5.6	1.20	0.88	0	1:12.1

* From Brandt & Muir (1971)

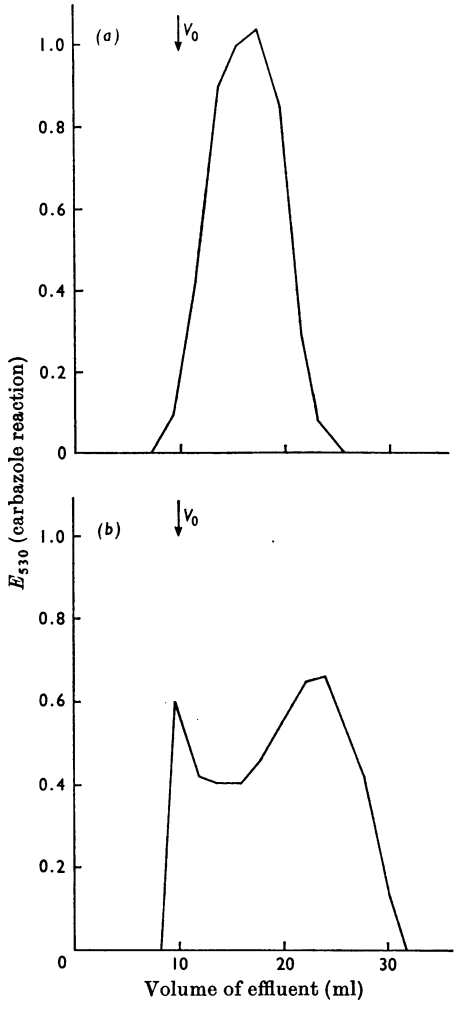


Fig. 3. Elution of protein–polysaccharides from a column (50cm×0.9cm) of Sepharose 4B with 0.5M-sodium acetate, pH6.5, at 4°C; the uronic acid content of each 0.9ml fraction was determined. V_0 designates the void volume of the column. (*a*) Extract S-Col; (*b*) extract S-CU.

Although uncorrected for losses on hydrolysis, serine, glutamic acid and glycine residues accounted for 43% of the amino acids.

Extract S-CU. The protein–polysaccharides extracted by urea after collagenase digestion of the residual cartilage contained 16.7% of protein (Table 1) and were excluded from 6% agarose but were eluted from Sepharose 4B as two peaks (Fig. 3*b*) each containing about the same amount of hydroxyproline (0.3%) and each having the same glucosamine/galactosamine molar ratio of 1:11. Treatment with 8M-urea before gel chromatography did not affect the elution profile; neither did removing the remaining hydroxyproline by further treatment with collagenase, both fractions being eluted in the same positions as before.

Table 2 shows that the amino acid composition of these protein–polysaccharides was unlike that of protein–polysaccharides in the previous extracts.

Alkaline β-carbonyl elimination. Losses of serine and also of threonine residues occurred when the protein–polysaccharides in the extracts were treated with alkali (Table 3), the highest proportionate loss of both hydroxyamino acids occurring with extract S-Col, although the total losses were 75 and 72 μmol/g respectively with extracts S-Col and S-CU (Table 4). Apart from some losses of basic amino acids (about 12%) there were no losses of other amino acids.

Carbohydrate chains. When calculated from the galactosamine/pentose molar ratios the average length of the chondroitin sulphate chains of the protein–polysaccharides in extracts S-Col and S-CU and that obtained after proteolysis of the cartilage residue was about 22–23 disaccharide units (Table 4).

Since the molar proportions of xylose and serine destroyed were about equal in the protein–polysaccharides of extracts S-Col and S-CU, and the fourth sodium acetate extract (Table 4), if all xylose groups are involved in linking chondroitin sulphate to protein as in protein–polysaccharides from bovine nasal septum (Lindahl & Rodén, 1966; Rodén & Armand, 1966), serine residues would be exclusively involved in linking chondroitin sulphate chains. The threonine that is also destroyed on treatment with alkali may be involved in linking keratan sulphate chains to protein. If so, it can be calculated from the glucosamine contents that the average length of keratan sulphate chains was 13, 12 and 10 disaccharide units in protein–polysaccharides of the fourth sodium acetate extract and extracts S-Col and S-CU respectively (Table 4).

DISCUSSION

Since the final residue (Res-CU; Scheme 1) contained only 2% of the uronic acid originally present, nearly all the protein–polysaccharides were ultimately brought into solution by the sequential extractions. Nevertheless, including the uronic acid recovered in the previous sodium acetate extracts (Brandt & Muir, 1971), 28% of the uronic acid of the tissue was not accounted for. This may be due to the large number of manipulations that had been carried out leading to substantial mechanical losses; including the treatment the cartilage had received previously (Brandt & Muir, 1971), altogether twelve manipulations had been carried out, excluding washings of residues.

Table 2. *Amino acid composition of protein–polysaccharides in fractions S-Col and S-CU compared with that of the protein–polysaccharide in the fourth sodium acetate extract of the same cartilage* (*see Brandt & Muir*, 1971)

Samples were hydrolysed in 6M-HCl at 105°C for 24h. No corrections were applied for losses during hydrolysis.

	Composition of extract S-Col		Composition of extract S-CU		Composition of protein–polysaccharides in fourth sodium acetate extract	
	(μmol/g)	(residues/1000 residues)	(μmol/g)	(residues/1000 residues)	(μmol/g)	(residues/1000 residues)
Asp	68	86	150	111	88	82
Thr	36	45	79	58	58	54
Ser	117	147	140	104	116	108
Glu	112	141	179	133	124	115
Pro	78	98	101	75	44	41
Gly	111	139	175	130	152	141
Ala	62	78	103	76	87	81
Cys	—	—	—	—	5	5
Val	43	54	92	68	70	65
Met	3	4	5	4	6	6
Ile	31	39	51	38	43	40
Leu	61	77	111	82	120	112
Tyr	1	1	27	20	24	22
Phe	24	30	41	30	38	35
Lys	7	9	21	16	24	22
His	12	15	16	12	28	26
Arg	28	36	53	39	50	46

Table 3. *Proportion of hydroxy amino acids destroyed on treatment of protein–polysaccharides with* 0.5M-*sodium hydroxide for* 24h *at* 4°C

Experimental details are given in the text.

Extract	Percentage of total destroyed	
	Serine	Threonine
4th sodium acetate extract	44	22
S-Col	55	31
S-CU	43	15

The gross appearance of the cartilage was little changed, however, even after collagenase digestion, although this was incomplete. Curtis & Klein (1963) showed that it is necessary to remove most of the collagen before there is any visible change in the appearance of cartilage.

Metachromasia is an unreliable guide in showing the survival of protein–polysaccharides in cartilage, as there was no difference in metachromasia even when almost half the total uronic acid of the tissue had been removed. Decrease in metachromasia became apparent only locally where collagen had been digested by collagenase. It would thus appear, as originally suggested by Schubert (1964), that protein–polysaccharides are enmeshed in the collagen network. Some of the protein–polysaccharides may also be bound in some way to the collagen. This is suggested by the failure to remove hydroxyproline from protein–polysaccharides in extract S-U until they had been degraded. There are two forms of collagen in cartilage of chick embryos, one of which is unique to this tissue (Miller & Matukas, 1969). If it is also present in adult mammalian cartilage it might have a particular affinity for protein–polysaccharides. The protein content of extract S-U was as high as 35.3% (Table 1) but about 40% of this was collagen, so that it resembled fractions known as PP-H isolated from bovine nasal septum (Pal & Schubert, 1965) and human costal cartilage (Rosenberg, Johnson & Schubert, 1965). Otherwise the protein–polysaccharides of extract S-U resembled those of the fifth sodium acetate extract in the galactosamine/glucosamine molar ratio (Table 1) and in size distribution, being partly excluded from Sepharose 4B (Brandt & Muir, 1971).

The protein content and amino acid composition of the protein–polysaccharides in the other extracts showed that they differed from each other and from those in the fourth and fifth sodium acetate extracts (Tables 1 and 2). Their sizes also varied (Fig. 3) but unlike the protein–polysaccharides in sodium acetate extracts (Brandt & Muir, 1971) there was no relationship of size with increasing difficulty of extraction. Thus extract S-Col was smaller and more uniform in size than most of the protein–polysaccharides in previous sodium acetate extracts (Brandt & Muir, 1971) being almost wholly retarded

Table 4. *Comparison of the amounts of hydroxy amino acids destroyed when protein–polysaccharides were treated with* 0.5M-*sodium hydroxide for* 24h *with the amounts present and molar ratios of glucosamine, galactosamine, hexose and pentose in the starting material*

Experimental details are given in the text.

	Hydroxy amino acids destroyed (μmol/g)		Sugar composition (μmol/g)			
Extract	Serine	Threonine	Glucosamine	Galactosamine	Hexose	Pentose
4th sodium acetate extract	51	13	170	1540	290	53
S-Col	64	11	133	1495	294	65
S-CU	60	12	122	1384	272	63

	Molar ratios		
	Galactosamine/pentose	Galactosamine/serine destroyed	Glucosamine/threonine destroyed
4th sodium acetate extract	29:1	30:1	13:1
S-Col	23:1	23:1	12:1
S-CU	22:1	23:1	10:1

on Sepharose 4B, giving a symmetrical elution profile (Fig. 3*a*), yet the protein content and glucosamine/galactosamine molar ratio were lower than those of compounds in the preceding sodium acetate extract (Table 1).

The protein–polysaccharides extracted after collagenase digestion showed no relationship of size with protein and glucosamine content as had those extracted with sodium acetate (Brandt & Muir, 1971). The protein–polysaccharides in extract S-CU contained much more protein than those in the preceding extract, S-Col, but there was no great difference in size (Fig. 3) and the glucosamine/galactosamine molar ratios were the same (Table 1). The larger and smaller components of extract S-CU, moreover, had the same protein contents and glucosamine/galactosamine molar ratios. Since the elution profiles were unchanged after the residual collagen had been removed with collagenase, the larger component was not formed of smaller ones held together by collagen.

It would seem unlikely that collagenase had cleaved peptide bonds in the protein–polysaccharides, since it did not affect the viscosity (Fig. 1) or elution profile of the protein–polysaccharides of the first sodium acetate extract. However, since the amino acid composition of protein–polysaccharides in different extracts varied, it is conceivable that some may possess a region having the correct amino acid sequence for attack by collagenase (Hannig & Nordwig, 1967), whereas others do not.

The variation in composition and size of protein–polysaccharides of cartilage has been discussed (Tsiganos & Muir, 1969; Brandt & Muir, 1971; Tsiganos, Hardingham & Muir, 1971) and it was concluded that it could not be explained by differences in lengths of the carbohydrate chains attached to the core protein nor solely by variation in the relative proportions of chondroitin sulphate and keratan sulphate. Even after dissociation and removal of a specific component inducing aggregation of protein–polysaccharides (Hascall & Sajdera, 1969), they remained heterogeneous in composition and size when fractionated according to their buoyant density in a caesium chloride gradient (Tsiganos *et al.* 1971), indicating that there were intrinsic differences between protein–polysaccharides of cartilage, in addition to variations due to their aggregation. The differences between the protein–polysaccharides in the extracts examined here (Tables 1 and 2) as well as those reported by Brandt & Muir (1971) further support this view, particularly since together, the protein–polysaccharides in these extracts represent most of those in the tissue. Insufficient material precluded studies of the heterogeneity by ultracentrifugation and polyacrylamide-gel electrophoresis is inapplicable because protein–polysaccharides do not penetrate the gel (C. A. McDevitt & H. Muir, unpublished work). Since other protein–polysaccharide preparations were similarly heterogeneous on gel chromatography as well as heterogeneous in buoyant density, the various fractions showing considerable differences in composition (Tsiganos *et al.* 1971), it would appear reasonable to assume that in this study also gel chromatography revealed real molecular heterogeneity.

It is unlikely, however, from the way that the protein–polysaccharides were prepared here, that they were dissociated from any aggregating com-

ponent. Nonetheless, the protein–polysaccharides were mostly of a size small enough to penetrate Sepharose 4B (Fig. 3), whereas those prepared as described by Hascall & Sajdera (1969) that have not been disaggregated are partially excluded even from Sepharose 2B (Tsiganos *et al.* 1971). This might suggest that the component that brings about aggregation was in some way affected by collagenase. The protein–polysaccharides did not show reversible disaggregation in 8M-urea, as had those in the fourth and fifth sodium acetate extracts of the same cartilage (Brandt & Muir, 1971).

The average length of chondroitin sulphate chains appeared to be shorter in extract S-Col than in protein–polysaccharides of previous sodium acetate extracts (Table 4), but the uronic acid contents were similar (Table 1). This would imply that extract S-Col contained more chains per unit weight, in agreement with the somewhat higher proportion of alkali-labile serine residues (Table 3).

Extracts S-Col and S-CU contained 11–15% more hexose than could be accounted for by that in the chondroitin sulphate linkage regions and that due to keratan sulphate, assuming this to consist of disaccharide repeating units. A similar discrepancy was noted earlier (Tsiganos & Muir, 1969), and although the colorimetric methods used here are somewhat inaccurate, no such discrepancy was noted by using the same methods in certain other protein–polysaccharides such as in a protein–polysaccharide isolated from Kurloff cells of the guinea pig that contained no keratan sulphate (Dean & Muir, 1970). The excess of hexose might therefore be due to some structural feature, such as the branching of keratan sulphate chains, as shown by Bhavanandan & Meyer (1968).

As assessed by their glucosamine/galactosamine molar ratios, the protein–polysaccharides more closely associated with collagen examined here contained less keratan sulphate than those more easily extracted with sodium acetate (Brandt & Muir, 1971). This might explain why in human femoral condyles the relative proportion of keratan sulphate to chondroitin sulphate tended to increase with depth from the articular surface (Maroudas, Muir & Wingham, 1969) whereas the collagen content decreased (Muir, Bullough & Maroudas, 1970). The molecular organization of cartilage matrix is thus considerably more complex than would be supposed from its histological appearance.

We are very grateful to Mr R. Ewins for excellent technical help. Mr W. Everett, of T. Walls and Sons Ltd., Atlas Road, London N.W.10, U.K., generously provided the tissue used in this study. We thank the National Cystic Fibrosis Research Foundation for their award of a Research Fellowship to K.B. and the Arthritis and Rheumatism Council for general support.

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