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Studies on Protein–Polysaccharides from Pig Laryngeal Cartilage

HETEROGENEITY, FRACTIONATION AND CHARACTERIZATION

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1. Protein–polysaccharides from pig laryngeal cartilage extracted by two procedures described in the preceding paper (Tsiganos & Muir, 1969) were shown to consist of macromolecules of various sizes as assessed by gel filtration in 4% and 6% agarose. 2. A larger proportion of the smaller molecules was present in the preparation obtained by brief extraction in iso-osmotic sodium acetate (procedure I) than in that obtained by more prolonged extraction in 10% (w/v) calcium chloride (procedure II). 3. Two fractions were separated by gel filtration in 6% agarose and by electrophoresis in compressed glass fibre. These fractions differed in chemical composition and in antigenic determinants. The gel-retarded fraction R and that of higher electrophoretic mobility possessed the same single antigen, whereas the gel-excluded fraction E and the slower electrophoretic fraction contained all the antigens of the starting material including that of fraction R. 4. Five *N*-terminal amino acid residues were identified in preparation I and fraction E, only two of which were present in fraction R. 5. The relative proportions of gel-excluded and gel-retarded fractions did not change when solutions of high ionic strength, urea or guanidine hydrochloride were used for elution. 6. The differences in chemical and amino acid composition between fractions R and E showed that the latter was not a simple aggregate of the former. Fraction E contained more basic and aromatic amino acids, and some methionine and cystine; the last two were absent from fraction R. Hydroxyproline was not detected in either fraction. 7. The number of glycosidic linkages in both fractions was estimated by alkaline β-elimination. Appreciable amounts of threonine as well as serine were destroyed in both fractions. An average chain length for chondroitin sulphate was calculated from the galactosamine content of both fractions and the amounts of hydroxy amino acid destroyed. Average chain lengths were also calculated from the xylose and galactosamine content of each fraction. Each independent method gave a value of approximately 28 disaccharide units for the chain length in both fractions and hence their difference in size could not be explained by differences in the length of carbohydrate chains. 8. All fractions contained glucosamine, which was attributed to keratan sulphate. Content of both protein and keratan sulphate increased with the size of the macromolecules. 9 It is suggested, from these results, that chondroitin sulphate–protein complexes normally exist as a heterogeneous population of macromolecules in cartilage, and that keratan sulphate is involved in the formation of larger molecules.

In the preceding paper (Tsiganos & Muir, 1969), where a definition of protein–polysaccharide is given, it was found that protein–polysaccharides of pig laryngeal cartilage consisted of several similar compounds distinguishable by their analytical differences and sedimentation behaviour. Moreover, it appeared that different extraction procedures selected different proportions of the molecules present in the tissues. The separation of the smaller constituents of such a mixture of protein–polysaccharides and their characterization by chemical and immunological methods is described in this paper. Preliminary reports of some of these results have appeared (Tsiganos & Muir, 1966*b*, 1967*a*,*b*,*c*,*d*; Tsiganos, 1968).

MATERIALS AND METHODS

All reagents were of analytical grade, except when otherwise stated, or as stated in the preceding paper (Tsiganos & Muir, 1969). For chemical analysis all samples were dried to constant weight at 80° *in vacuo*.

Analytical methods

Hexuronic acid. The method of Bitter & Muir (1962) was used, with glucuronolactone as the standard.

Hexosamine. The hydrolytic conditions used and the modification of the procedure of Cessi & Piliego (1960) were as described in the preceding paper (Tsiganos & Muir, 1969). The standard was glucosamine hydrochloride, recrystallized to constant optical rotation.

Molar ratios of glucosamine to galactosamine. The molar ratios were determined either as described in the preceding paper (Tsiganos & Muir, 1969) or with the use of the 15cm. column of the Locarte amino acid analyser at 56°, eluted with 0·12M-citrate buffer, pH5·28. The colour yields of standards of galactosamine and glucosamine in the ninhydrin reaction were determined for each batch of ninhydrin and the resulting correction factor was applied to account for the lower colour yield of galactosamine. The very small amounts of glucosamine in some samples were estimated by the use of the scale expansion of the Kent recorder.

Hexose. The method of Trevelyan & Harrison (1952) was used, with galactose as standard. To account for interference by the large amounts of uronic acid and hexosamine in the samples, approximately similar amounts of glucuronolactone and hexosamine (glucosamine) were added to the control tubes, and to tubes containing 25μg. of galactose/ml.

Xylose. The anthrone method of Tsiganos & Muir (1966*a*) was used, with xylose as standard. Interference by other sugars in the samples was accounted for by adding to the control tubes galactose, glucuronolactone and hexosamine (glucosamine) in amounts similar to those in the samples.

Sulphate. After hydrolysis of the samples in 60% (v/v) formic acid at 100° for 8hr., sulphate was determined as described by Muir & Jacobs (1967) by using a modification of the method of Jones & Letham (1954); K_2SO_4 was used as a standard.

Nitrogen. Total nitrogen was determined by the indanetrione hydrate method of Jacobs (1962).

Amino acids. Samples of protein–polysaccharide of known weight (about 9mg.) were hydrolysed in 18ml. of 50% (v/v) conc. HCl. O_2-free N_2 was bubbled through the solution for 15min. before the containers were sealed and heated at 105° for 24hr. The hydrolysates were filtered through Whatman no. 50 paper, which was washed several times with deionized water. Filtrate and washings were combined and evaporated to dryness in a rotary evaporator at 40–45° (Moore & Stein, 1963). Excess of acid was removed by repeated evaporation after addition of water. Drying of the hydrolysates was complete within 2hr. The oxidation of cysteine to cystine was completed as recommended by Moore & Stein (1963) by dissolving the hydrolysates in 0·8ml. of 0·2M-potassium phosphate buffer, pH6·5, and keeping them at room temperature for 4hr. before again evaporating to dryness. The hydrolysates were dissolved in a known volume of starting buffer, pH2·8, and 0·5ml. portions were used to obtain duplicate analyses with a Technicon amino acid analyser. The column (140cm. × 0·6cm.) was packed with resin type A (particle size 22μm.), and maintained at 60°. The recommended Technicon buffer systems in a multi-chamber device (Autograd) were used except that the pH of the starting buffer was lowered to 2·80 in order to separate the amino sugars from the rest of the amino acids. In this system glucosamine preceded galactosamine and both were eluted between alanine and valine. This separation, however, was not always reproducible and galactosamine was then eluted with valine, possibly because of small variations in the pH. When this occurred a value for valine content was obtained on a second run, by altering the pH of the starting buffer to 3·15, with the result that glucosamine followed methionine and galactosamine was eluted with isoleucine, for which a value had already been obtained. Serine was adequately separated from threonine by replacing 2ml. and 1ml. of the buffer in chambers 1 and 2 by the same volumes of methanol respectively. An internal standard of 0·2μmole of norleucine was included in each analysis, which required about 21hr. The Technicon standard mixture of amino acids was used to identify and determine individual amino acids by the method of Spackman, Stein & Moore (1958). Most of the ninhydrin-positive artifacts formed from hexosamine during hydrolysis in the presence of uronic acid (Anderson, Hoffman & Meyer, 1965; Muir & Jacobs, 1967) were eluted near the front of the chromatogram, although not at the position of hydroxyproline. In addition there was one small peak preceding lysine.

Alkaline β-carbonyl elimination. A known weight (about 9mg.) of retarded and excluded fractions from 6% agarose was dissolved in 4·5ml. of 0·5M-NaOH and kept under N_2 at 18–20° for 19hr. (Anderson *et al.* 1965), after which 9ml. of conc. HCl and 4·5ml. of deionized water were added. Hydrolysis was then carried out as described for amino acids.

In another experiment 50mg. of material from procedure I was dissolved in 0·5M-NaOH and kept under N_2 at 18–20° for 40hr. The formation of double bonds was assessed by titration with bromine water as follows: duplicate 1ml. samples removed at zero time and intervals thereafter were treated with 0·1ml. of 2·5M-H_2SO_4 followed by 3ml. of a solution of 0·25% (v/v) bromine in 20% (v/v) acetic acid. The samples were stored in the dark for 10min. at 18–20°, before 2ml. of 15% KI was added and the liberated I_2 was titrated with 0·5mM-$Na_2S_2O_3$ with starch as the indicator (Kolthoff & Sandell, 1958). Alkali-treated chondroitin sulphate–peptide (Muir & Jacobs, 1967) was subjected to the same procedure and this solution was used as a blank in the titrations.

Thiol groups. Thiol groups were determined by using 5,5′-dithiobis-(2-nitrobenzoic acid), as described by Ellman (1959), with some modifications. Samples (about 5mg.) were used dissolved in 3ml. of tris–acetate buffer, pH8·0, containing 0·3ml. of a 5% (w/v) solution of sodium dodecyl sulphate (Dr J. Cebra, personal communication). Crystalline egg albumin was used as a test compound.

N-Terminal amino acids. Dinitrophenylation was carried out by the method of Durant, Hendrickson & Montgomery (1962). Approx. 200mg. of the original mixture of protein–polysaccharides or 70mg. of separated fractions was dissolved in 20ml. of 0·2M-H_3BO_3, the solution was adjusted to pH7·8 with 0·05M-$Na_2B_4O_7$, and 10ml. of ethanol containing 1ml. of 1-fluoro-2,4-dinitrobenzene was added, when the pH rose to 8·5. The mixture was stirred for 20hr. at room temperature (18–20°) in the dark under N_2. At intervals, the pH was adjusted to 8·5 with $Na_2B_4O_7$. After extraction of the solution with ether, the protein–polysaccharides were precipitated with 4vol. of ethanol containing sodium acetate, and washed by suspending in ethanol and centrifuging, until the supernatant was colourless. They were then dissolved in 5–7ml. of water and

precipitated with ethanol, washed and dried as described in the preceding paper (Tsiganos & Muir, 1969).

The entire samples of dinitrophenylated protein–polysaccharides were dissolved in 5ml. of deionized water and added to a thin slurry of about 30g. of wet Dowex 50 (H^+ form; 8% DVB) resin (Steven, 1962) in a long-necked flask. The air was displaced with N_2 and the flask sealed and heated at 100° while being rotated at 80rev./min. The suspension was then filtered and the resin washed several times with boiling water and finally with aq. 0·8M-NH_3. The filtrate and water washings were combined, filtered through Whatman no. 41 paper, concentrated by rotary evaporation to about 5ml. and acidified with 1M-HCl. The aq.-NH_3 washings were treated likewise.

DNP-amino acids were extracted with peroxide-free ether, which was washed once with water, before being evaporated *in vacuo*, and the residues were taken up in the minimum volume of acetone (Fraenkel-Conrat, Harris & Levy, 1955). Light was excluded as far as possible during the manipulations. The samples of ether-soluble DNP-amino acids were compared with a standard mixture by two-dimensional t.l.c. with glass plates (20cm. × 20cm.) covered with a layer (0·25mm. thick) of silica gel (Chromalay; May and Baker Ltd., Dagenham, Essex). Toluene–pyridine–ethylene chlorohydrin–0·8M-NH_3 (10:3:6:6, by vol.; upper phase) was used in the first direction and chloroform–benzyl alcohol–acetic acid (70:30:3, by vol.) in the second (Brenner, Niederwieser & Pataki, 1965). The yellow spots absorbing in the ultraviolet were shown to contain no free α-amino groups when sprayed with a 0·3% (w/v) solution of ninhydrin in butan-1-ol containing 3% of acetic acid and heated at 110° for 10min. (Waldi, 1965). To confirm the identification of serine and threonine, the respective spots were scraped off the plates and the DNP-amino acids extracted with chloroform–acetic acid (99:1, v/v), dried and then rechromatographed with the appropriate standard DNP-amino acid.

The aq.-NH_3 washings contained no α-DNP-amino acids as shown by t.l.c. The aqueous phase, after extraction with ether, was concentrated by rotary evaporation and subjected directly to one-dimensional t.l.c. with propan-1-ol–34% (v/v) NH_3 (sp.gr. 0·88) (7:3, v/v) (Brenner *et al.* 1965). Only ε-DNP-lysine but no α-DNP-amino acids were found.

Gel-filtration procedures

Dry particles were allowed to swell in water containing merthiolate (1:10000, w/v) for 48hr. at room temperature and the remaining fine particles removed by decantation. The gels were then collected by filtration and suspended in the solution to be used for elution and allowed to equilibrate overnight. After deaeration, a thin slurry was used to pack columns of various sizes as described by Flodin (1962). Each column was washed with twice its bed volume of eluent, passed through at the same flow rate as used in the fractionation. To eliminate 'wall effect' (Hjertén & Mosbach, 1962) the columns were previously treated with 2% (v/v) dichlorodimethylsilane in carbon tetrachloride (silicone Repelcote; Hopkin and Williams Ltd., Chadwell Heath, Essex). Columns of agarose gels were packed and run at 4°, and those of Bio-Gel and Sephadex at room temperature.

The void volume, V_0, for each column was determined with haemocyanin obtained from *Helix pomatia* (Heirweigh, Borginon & Loutie, 1961); when urea or guanidine was used, a solution of Blue Dextran 2000 [Pharmacia (G.B.) Ltd., London W.13] was employed.

The samples were dissolved at 4° overnight with stirring and the effluent fractions were analysed for uronic acid by the carbazole method (Bitter & Muir, 1962).

Fractions corresponding to the void volume were pooled and concentrated by ultrafiltration under reduced pressure by using $\frac{8}{32}$in. dialysis tubing previously heated at 97° for 72hr. to decrease its porosity (Callanan, Carrol & Mitchell, 1957). The retarded fractions were pooled and treated likewise, and the protein–polysaccharides in both fractions precipitated with ethanol, washed and dried as described in the preceding paper (Tsiganos & Muir, 1969). The isolated fractions were run once more and the protein–polysaccharides were isolated as before.

To ascertain the amount of protein–polysaccharide that was retarded and excluded by the different gels, the uronic acid contents of the respective pooled fractions were compared.

Bio-Gel P-300. Dry bead polymerized Bio-Gel P-300 (60–150 mesh from Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was sieved so that particles passing through sieve no. 60 and retained by sieve no. 240 were used. Columns of 2cm. (internal diam.) and of lengths to give bed volumes 400–500ml. were used. Elution was performed with 0·1M- and 1M-NaCl, pH6·8, 0·1M- and 1M-$MgSO_4$, pH7·2, and 7M-urea, pH7·2. Samples containing 10–15mg. in 4ml. were applied and 5ml. fractions collected at the rate of 20ml./hr.

Sephadex G-200 (*Pharmacia Ltd.*; 40–120 μm.). A column 162cm. × 2cm. was used. Samples (15–20mg.) in 5ml. were applied and elution with 0·1M- and 1M-$MgSO_4$, pH7·2, was performed. Fractions (10ml.) were collected at the rate of 10ml./hr.

Agarose. Agarose was precipitated from hot aqueous solutions of agar (Ionagar no. 2; Oxoid Ltd., London E.C.4) with polyethylene glycol 6000 (BDH Chemicals Ltd., Poole, Dorset) (Russel, Mead & Polson, 1964). After reprecipitating twice more it was almost free from agaropectin. Beads of 6% and 4% agarose were prepared essentially by Hjertén's (1964) method with some modifications (Tsiganos, 1968). Coarse particles were removed by wet sieving and fines by decantation. Finally, particles of size 75–125μm. were separated by the elutriation procedure described in the preceding paper (Tsiganos & Muir, 1969), with the flow rate of water adjusted to about 100ml./100sec.

Columns of three sizes were used: (*A*) 52cm. × 1·4cm. packed with 4% agarose and eluted with 0·5M-sodium acetate, pH6·8. Samples (8–10mg.) in 2ml. were applied and 2ml. fractions collected at 8ml./hr. (*B*) 42cm. × 2·2cm. packed with 6% agarose and eluted with 0·1M- and 1M-NaCl, pH6·8, 0·1M- and 1M-$MgSO_4$, pH7·2, 1M-$MgSO_4$, pH3·0, and 4M-guanidine acetate, pH6·5. Samples (10–15mg.) in 2ml. were applied and 4ml. fractions were collected at 16ml./hr. (*C*) 110cm. × 2·8cm. packed with 6% agarose and eluted with 0·5M-sodium acetate, pH6·8. Samples (about 50mg.) in 7ml. were applied and 7ml. fractions collected at 30ml./hr.

The isolated protein–polysaccharides from the retarded and excluded fractions from column (*C*) were re-run once more, and isolated as described above. To avoid contamination of the protein–polysaccharides with soluble components from the dialysis tubing the protein–polysaccharides were

finally precipitated with 9-aminoacridine and isolated as described in the preceding paper (Tsiganos & Muir, 1969).

Preparative zone electrophoresis

Electrophoresis in compressed glass fibre was carried out as described by Muir & Jacobs (1967) with 150mg. of sample. The material was arbitrarily separated into fractions of higher and lower mobility (F_I and S respectively), and the molar ratio of glucosamine to galactosamine was determined in each. The material of higher mobility (F_I) was again subjected to electrophoresis and the faster moving fraction separated (F_{II}). Fraction F_{II} was subjected to electrophoresis a third time and the faster moving fraction again separated (F_{III}). After every electrophoresis a portion of each faster-moving fraction was taken for the determination of the molar ratio of glucosamine to galactosamine. The fractions obtained from the first electrophoresis (S and F_I) were also examined on 6% agarose eluted with 0·5M-sodium acetate to ascertain the relative proportions of retarded and excluded materials.

Immunization procedure

Antibodies against preparation I and glycoprotein fractions were raised in outbred New Zealand white rabbits. A 3mg. portion of each sample in 0·5ml. of 0·15M-NaCl was fully emulsified with 0·5ml. of Freund's complete adjuvant and injected into a hind foot-pad of each rabbit (Loewi & Muir, 1965). One week later and 1 month thereafter similar injections were given subcutaneously at the back of the neck. One week after the last injection about 20ml. of blood was removed from the ear vein. The sera were separated by centrifuging and heating at 56° for 1hr. to inactivate complement (Kabat & Mayer, 1961) and then stored frozen.

Ouchterlony double gel diffusion

The presence of precipitating antibodies was examined by double gel diffusion (Ouchterlony, 1948) in 1% agar (Ionagar no. 2) in barbitone–saline buffer, pH7·2 (Oxoid complement-fixation diluent tablets), containing merthiolate, 1:10000 (w/v). Solutions of the antigens in the same buffer at concentrations of 0·5–5mg./ml. were placed in circumferential wells and diffused against individual antisera placed in a central well. The plates were kept in a humid atmosphere at 4° or at room temperature for up to 1 week. In parallel experiments, before being placed in the wells the protein–polysaccharides were first digested with testicular hyaluronidase (EC 3.2.1.35) (Loewi & Muir, 1965) at 37° for 16hr. (1500units/10mg. dissolved in 0·1M-acetate–0·15M-NaCl buffer, pH6·0; Hoffman, Meyer & Linker, 1956). In other experiments, before diffusion the protein–polysaccharides were digested with Pronase from *Streptomyces griseus* (Kaken Chemical Co., Tokyo, Japan) in 0·1M-borate buffer, pH8·2, containing 0·02M-$CaCl_2$ (Nomoto & Narahashi, 1959) at 60° for 5hr.

RESULTS

Protein–polysaccharides obtained by the two procedures of Tsiganos & Muir (1969) were compared by gel filtration. Table 1 shows that a higher proportion of material from procedure I (iso-osmotic neutral sodium acetate) was retarded by 6% agarose than that from procedure II (neutral 10% calcium chloride), indicating that more larger molecules were extracted by procedure II than by procedure I. Table 1 also shows that gels of increasing porosity from Sephadex G-200 to 4% agarose retained increasing proportions of material from procedure I, suggesting that a range of sizes of molecules was present in preparation I. Recoveries based on uronic acid were complete, and on repeating the gel filtration through 6% agarose the small (10%) contamination by the other fraction that remained was removed, as shown by the elution profile of the third run (Fig. 1). The larger molecules in the excluded fraction E were not all of the same size either, since 20% of this fraction was retained by 4% agarose (Table 1). 7M-Urea, 4M-guanidine and salt solutions of high ionic strength or low pH did not affect in any way the elution profiles of either preparation I or II. Fractions containing a greater proportion of larger molecules had higher relative amounts of glucosamine (Table 2) and protein (Table 3). The analyses of each main fraction from 6% agarose after re-running once are shown in Table 3. The excluded fraction E not only contained more glucosamine (Table 2) and more protein than fraction *R* but also more hexose in relation to glucosamine. The molar ratio of hexose to glucosamine was about 5:1 in fraction R and about 3:1 in fraction E. The latter had rather more sulphate than fraction E. The analyses accounted for 97% and 91% of the dry weight of fractions R and E respectively, assuming all anionic groups to be combined with Na^+.

Table 1. *Protein–polysaccharide retarded by various gels*

Preparation I was obtained by brief extraction with 0·15M-sodium acetate, pH6·8, preparation II by prolonged extraction with 10% $CaCl_2$, pH6·8. Fraction E was that excluded by 6% agarose. Electrophoretic fractions were arbitrarily divided into slower (S) and faster (F) fractions (see the text).

	Protein–polysaccharide retarded (% of total uronic acid)			
	Sephadex G-200	Bio-Gel P-300	6% Agarose	4% Agarose
Preparation I	8	22	45	58
Preparation II			24	
Fraction E			0	20
Electrophoretic fraction S			40	
Electrophoretic fraction F			75	

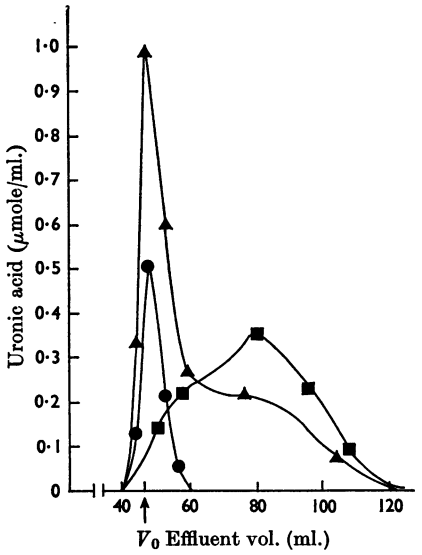


Fig. 1. Elution of protein–polysaccharides (determined as uronic acid) with 0·5M-sodium acetate, pH 6·8, at 4° from a column (42cm. × 2·2cm.) of 6% agarose. Fractions (4ml.) were collected. ▲, Preparation I; ●, excluded fraction E; ■, retarded fraction R, after being rechromatographed once (see the text).

Table 2. *Molar ratios of glucosamine to galactosamine of protein–polysaccharides*

The specimens were the same as those of Table 1, and also included fraction R, which was retarded by 6% agarose, and the faster electrophoretic fraction F_I, which was rerun once (F_{II}) and twice (F_{III}) (see the text).

Specimen	Molar ratio
Preparation I	1:26
Preparation II	1:14
Fraction E	1:18
Fraction R	1:32
Electrophoretic fraction S	1:15
Electrophoretic fraction F_I	1:37
Electrophoretic fraction F_{II}	1:63
Electrophoretic fraction F_{III}	1:65

Separation of molecules of smaller size and lower molar ratio of glucosamine to galactosamine was also achieved by preparative electrophoresis in glass fibre. Thus, although discrete bands were not obtained, smaller molecules tended to segregate in the faster-moving fraction, because the proportion of this fraction retarded by 6% agarose rose from 45% to 75% (Table 1) and the molar ratio of glucosamine to galactosamine decreased from 1:26 to 1:37 (Table 2). On further electrophoresis this ratio reached an apparently limiting value of 1:65 (Table 2).

Table 3. *Chemical composition of agarose 6% fractions E and R*

	Chemical composition (g./100g. dry wt.)	
	Fraction E	Fraction R
Hexosamine (free base)	30·5	33·5
Glucuronic acid (lactone)	27·0	28·5
Protein*	7·2	5·3
Hexose (galactose†)	5·2	4·6
Xylose†	0·8	1·0
Sulphate	12·55	14·75
Total nitrogen	3·670	3·470
Hexosamine N (calc.)	2·385	2·620
Amino acid‡	1·127	0·840

* The sum of amino acid residues; (Table 4).
† Identified chromatographically (Tsiganos & Muir, 1969).
‡ Calculated as for protein.

The amino acid analyses from which protein contents were calculated are given in Table 4, uncorrected for losses on hydrolysis. Nonetheless, 99·8% of the total nitrogen was accounted for by these amino acid analyses together with the hexosamine contents in the retarded fraction R, and 96% in the excluded fraction E.

The main difference in amino acid compositions was that fraction E, of larger molecular size, contained substantially more aromatic and basic amino acids, as well as some sulphur-containing ones not detectable in fraction R. Cysteine could not be detected in either fraction by the colorimetric method of Ellman (1959). Serine, glutamic acid, glycine and proline were prominent in both fractions, there being approximately equal amounts of the first three in fraction E, and of serine and glycine in fraction R, and together these accounted for 35% of all amino acids. Hydroxyproline was not found in either fraction.

The proportion of hydroxy amino acids engaged in glycosidic linkages to carbohydrate chains, assessed by losses after alkaline β-carbonyl elimination, was somewhat greater in fraction R than in fraction E (Table 5). Losses of threonine as well as of serine were found. Thus 25% of threonine and 56% of serine in fraction E were destroyed, and 40% of threonine and 63% of serine were destroyed in fraction R. After 19hr. the β-elimination should have been completed, since bromine titres remained unchanged from 18 to 40hr. There were no losses of aspartic acid, glutamic acid, proline, alanine or valine on treatment with alkali. Losses ranging from 5 to 10% were observed for all other amino acids.

Table 4. *Amino acid composition of 6%-agarose fractions E and R*

Duplicate analyses are expressed as μmoles/g. of protein–polysaccharides and mean residues/1000 residues. Samples were hydrolysed in 6M-HCl at 105° for 24hr. No corrections were applied for losses during hydrolysis.

	Composition of fraction E			Composition of fraction R		
	(μmoles/g.)		(residues/1000 residues)	(μmoles/g.)		(residues/1000 residues)
Hyp	0·0	0·0	0·0	0·0	0·0	0·0
Asp	51·3	53·5	74·0	30·0	30·3	56·1
Thr	41·5	40·8	58·1	19·9	21·4	38·4
Ser	91·5	93·2	130·4	95·0	88·6	170·7
Glu	89·7	91·3	127·9	74·9	77·9	142·1
Pro	71·8	65·4	96·9	56·8	55·5	104·5
Gly	94·5	93·0	132·8	87·4	92·3	167·0
Ala	49·6	50·5	70·7	36·0	34·6	65·7
CyS	~3	~3	~4		Not detectable	
Val	42·5	42·5	60·0	28·4	27·5	52·1
Met	~3	~3	~4		Not detectable	
Ile	25·2	26·0	36·2	29·6	30·0	55·4
Leu	60·7	61·6	86·2	40·5	42·1	77·8
Tyr	13·0	13·3	18·5	3·6	3·6	6·7
Phe	24·2	23·5	33·6	8·6	10·0	17·3
Lys	15·6	16·0	22·3	4·5	5·6	9·3
His	7·9	8·0	11·3	3·4	4·9	7·8
Arg	23·5	22·0	31·2	15·3	15·9	29·0

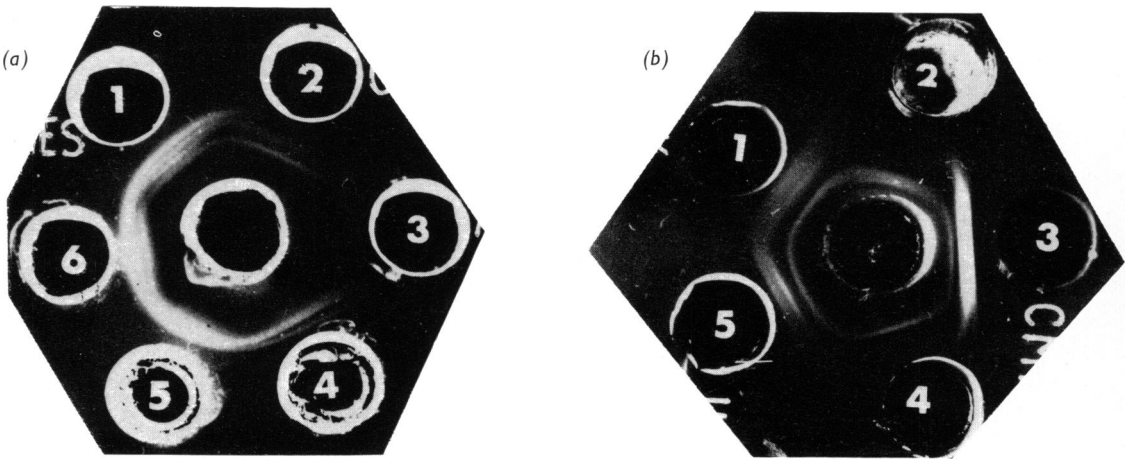
Table 5. *Hydroxy amino acid content of fractions E and R before and after treatment with 0·5M-sodium hydroxide for 19hr. at room temperature*

Duplicate analyses were carried on before (*a* and *c*) and after (*b* and *d*) the treatment.

	Hydroxy amino acid content (μmoles/g.)							
	Fraction E				Fraction R			
	(*a*)		(*b*)		(*c*)		(*d*)	
Thr	41·5	40·8	31·4	30·8	19·9	21·4	12·4	12·2
Ser	91·5	93·2	40·4	40·6	95·0	88·6	34·6	32·6

If it is assumed that all chondroitin sulphate (Lindahl & Rodén, 1966) and keratan sulphate (Seno, Meyer, Anderson & Hoffman, 1965) is linked to hydroxy amino acid by *O*-glycosidic bonds, then, as β-elimination appeared to be complete, the total amount of serine and threonine destroyed (Table 4) will give an estimate of the total number of chains of polysaccharide. If all glucosamine were derived from keratan sulphate with an average mol.wt. 10000 (Mathews & Cifonelli, 1965), the number of chondroitin sulphate chains may be obtained by difference. Disregarding the possibility that a small proportion of galactosamine might be derived from the linkage region of keratan sulphate (Bray-Anderson, Lieberman & Meyer, 1967), the average chain length of chondroitin sulphate was estimated to be about 28 disaccharide units in both fractions E and R. When the sugars from the linkage region (Lindahl & Rodén, 1966; Rodén & Armand, 1966) were added to this, the average weight of a chain of chondroitin sulphate (sodium salt) in both fractions was about 14500. These number-average estimates are similar to a value of M_n 13300 for alkali-prepared chondroitin sulphate from pig costal cartilage (Marler & Davidson, 1965), although the weight-average value for pig chondroitin sulphate–peptide was somewhat higher (M_w 19200; Muir & Jacobs, 1967).

The average weight of a chain of chondroitin sulphate may also be calculated from the molar proportion of xylose to galactosamine, if it is assumed that all chondroitin sulphate chains are attached to protein through xylose residues as in bovine nasal PP-L (Lindahl & Rodén, 1966; Rodén & Armand, 1966). The method used to determine xylose (Tsiganos & Muir, 1966*a*) was



EXPLANATION OF PLATE 1

Ouchterlony double-diffusion plates in agar. (*a*) The central well contained rabbit antiserum to protein–polysaccharides of preparation I. Preparation I at different concentrations (see the Materials and Methods section) after digestion with hyaluronidase *in vitro* was placed in the circumferential wells 1, 2, 4, 5 and 6 and, after digestion with Pronase, in well 3. (*b*) The central well contained the same antiserum as in (*a*). The circumferential wells contained the following samples: 1, electrophoretic fraction F_I: 2, electrophoretic fraction F_{II}; 3, protein–polysaccharides of preparation I; 4, fraction R; 5, fraction E. All samples were digested with hyaluronidase *in vitro* before diffusion.

specific and interference by other sugars was allowed for. A similar value of about 15000 was thus derived for the average chain weight in both retarted and excluded fractions.

Antigenic differences between fractions R and E were found on double diffusion with antisera raised against the unfractionated material. Digestion with hyaluronidase before applying the sample to the wells in the Ouchterlony plates was necessary to reveal multiple lines in fraction E (Plate 1), which showed the same number of precipitin lines as the unfractionated material and also when diffused against its own antiserum. Fraction R on the other hand produced only a single line with or without prior digestion with hyaluronidase when diffused against antisera to the unfractionated material or against its own antiserum. The fraction excluded by 4% agarose, like fraction E, showed all precipitin lines of the starting material, including one that showed identity with that of the retarded fraction R. Neither alkali-prepared chondroitin sulphate nor Pronase-digested protein–polysaccharide produced precipitin lines against the antisera to the unfractionated material, confirming earlier findings (Loewi & Muir, 1965) that the antigenicity of protein–polysaccharides lies in the protein moiety. Precipitin lines were not obtained when hyaluronidase alone was diffused against the antisera. Preparations I and II were free of serum proteins, since antibodies to pig serum raised in rabbits did not produce precipitin lines when diffused against each preparation. This enabled unabsorbed antisera to be used, avoiding possible losses of minor components.

When electrophoretic fractions were compared, the slower-moving fraction (S), like fraction E, showed all the precipitin lines of the starting material whereas the fraction of higher mobility after the second (F_{II}) and third electrophoresis (F_{III}) showed only a single precipitin line, in agreement with the findings of Loewi & Muir (1965). When diffused on the same Ouchterlony plate this line showed identity with the line of fraction R, and hence the same antigenic determinant was present in fraction R and the fraction of higher electrophoretic mobility. It is noteworthy that both these fractions had a very low ratio of glucosamine to galactosamine (Table 2).

Differences were found in the *N*-terminal amino acids of fractions R and E, as shown in Table 6. The unfractionated material (preparation I) and fraction E had the same five *N*-terminal amino acids, whereas fraction R, of smaller size, possessed only two of these five, namely glutamic acid and serine.

In the ultracentrifuge, three components were observed when preparations I and II were sedimented in 4M-guanidine hydrochloride (Tsiganos & Muir, 1969). Fraction R, which had a symmetrical boundary, corresponded to the most slowly sedimenting component. Fraction E, which separated into two, corresponded to the remainder (R. Pain, C. P. Tsiganos & H. Muir, unpublished work).

Table 6. *N-Terminal amino acid residues of preparation I and of fraction E and fraction R, identified as their DNP derivatives*

Amino acids	Preparation I	Fraction E	Fraction R
Glu	+	+	+
Ser	+	+	+
Gly	+	+	–
Ala	+	+	–
Val	+	Trace	–

DISCUSSION

Fraction R constituted approximately 45% of preparation I and appeared to be fairly homogeneous in the ultracentrifuge (R. Pain, C. P. Tsiganos & H. Muir, unpublished work). However, only about 22% was retarded by Bio-Gel P-300 (Table 1), so that the range of molecular sizes in fraction R is presumably distributed around the exclusion limits of Bio-Gel P-300, which for gum arabic (a polyuronide) is less than 300000 daltons (Anderson & Stoddart, 1966). A similar protein–polysaccharide fraction isolated by electrophoresis from an extract comparable with preparation II had M_w 230000 (Muir & Jacobs, 1967). Polysaccharides with expanded structures are eluted in advance of globular proteins of comparable molecular weight (Andrews, 1965; Anderson & Stoddart, 1966), owing to their larger effective hydrodynamic radii (Laurent & Killander, 1964; Squire, 1964; Siegel & Monty, 1966).

As protein–polysaccharides are rather slowly rehydrated, to ensure complete dissolution they were dissolved overnight with stirring, and, since recoveries from gel filtration were complete, the elution profiles were due to genuine differences in molecular size. The amounts in each pooled fraction were calculated from the uronic acid content and not from dry weights, the difference in protein content (Table 3) being small enough to justify such a comparison.

It seemed unlikely that fraction E consisted of aggregates of smaller molecules formed by electrostatic or hydrogen bonds, because the elution profiles were unchanged on elution with salts of high ionic strength, or with solutions of urea or guanidine, which dissociate large aggregates of protein–polysaccharide light fraction or PP-L (Mathews & Lozaityte, 1958). Further, when fraction E, which was prepared in 0·5M-sodium acetate, was

sedimented in 4 M-guanidine hydrochloride no slowly sedimenting material appeared that was comparable with fraction R (R. Pain, C. P. Tsiganos & H. Muir, unpublished work). In addition, only small amounts of material that was retarded by 6% agarose were produced from preparation I on electrophoresis, which should have dissociated aggregates formed by electrostatic interaction.

Fraction E was not a simple polymer of fraction R, because they had immunological and analytical differences (Plate 1 and Table 3) as well as differences in amino acid composition (Table 4). Hydrolysis was carried out under conditions which minimize losses, and 96–98% of the total nitrogen was accounted for by the analyses.

The demonstration of several precipitin lines (Plate 1) and five *N*-terminal amino acids (Table 6) indicates that preparation I contained several polypeptides, two of which were partially separated by gel filtration, since fraction R contained one of the antigens and two of the five *N*-terminal groups of fraction E (Plate 1 and Table 6). PP-L from bovine nasal septum, although homogeneous on ultracentrifugation, likewise appears to contain several polypeptides, as judged by molecular-weight determinations before and after digestion with hyaluronidase (Luscombe & Phelps, 1967) and by the presence of several *N*-terminal amino acids (Serafini-Fracassini, Peters & Floreani, 1967), although Hoffman, Mashburn & Meyer (1967*b*) consider that it contains a single core protein. Indeed, a number of components have been isolated by centrifugation in density gradients (Franek & Dunstone, 1967) and in solutions of high ionic strength (Pal, Doganges & Schubert, 1966; Rosenberg, Schubert & Sandson, 1967). It is possible, however, that some of this heterogeneity may have arisen from the action of proteolytic enzymes that are present in some PP-L preparations (Partridge, Whiting & Davies, 1965; Dziewiatkowski, Tourtellotte & Campo, 1967; Serafini-Fracassini *et al.* 1967). This does not account for fraction R, however, for three reasons. First, fraction R is unlikely to have arisen from random proteolysis because it contained fewer *N*-terminal amino acids than the starting material (Table 5). Secondly, preparation I, which involved a short extraction, contained more fraction R than did preparation II (Table 1), where extraction was prolonged and proteolysis more likely. Smaller molecules should be extracted more readily than larger ones during a very short period of extraction. Thirdly, fraction R contained only one of the several antigens of fraction E, which possessed all those of the starting material. Calcium chloride has a lyotropic effect on collagen and the appearance of larger molecules in preparation II suggests that these may be more closely associated with collagen, which may lead to local topographical variations in the distribution of different protein–polysaccharides, as is apparent in articular cartilage (Stockwell & Scott, 1967; Maroudas, Muir & Wingham, 1969).

That fraction E shares one of its antigens with fraction R suggests either that protein–polysaccharides with this antigen vary greatly in size, or that one of the several antigens in larger protein–polysaccharides is common to fraction R. This antigenic site might perhaps possess one of the two *N*-terminal amino acids of fraction R. If all glucosamine is attributable to keratan sulphate, the presence of glucosamine in all fractions suggests similarly either that protein–polysaccharides containing keratan sulphate had a very wide range of sizes or that keratan sulphate and chondroitin sulphate were each attached to the same protein core, the larger compounds containing relatively more keratan sulphate. Both chondroitin sulphate and keratan sulphate are attached to the same protein core in preparations from pig cartilage (Tsiganos & Muir, 1967*d*) as well as from other sources (Seno *et al.* 1965; Heinegård & Gardell, 1967), in agreement with a suggestion from earlier findings (Partridge, Davis & Adair, 1961). On the other hand, some chondroitin sulphate–proteins may contain no keratan sulphate, since Muir & Jacobs (1967) obtained an electrophoretic fraction of higher mobility, low protein content, possessing the single species-common antigen and virtually free of glucosamine. When samples were re-run, the proportion of glucosamine in the arbitrarily separated fractions (F_{II} and F_{III}) decreased (Table 2), indicating that compounds essentially free of keratan sulphate were being enriched in these fractions. Smaller molecules, which also contained somewhat more sulphate (Table 3; Muir & Jacobs, 1967), would have higher mobilities in compressed glass fibre (Table 1). Hoffman, Mashburn, Meyer & Bray (1967*a*) also found that compounds of higher electrophoretic mobility were richer in chondroitin sulphate and contained less protein. Since F_{II}, F_{III} and the gel-retarded fraction R shared a common antigen, and since fraction R contained two polypeptides, as judged by the presence of two *N*-terminal amino acids, one of these polypeptides should be common to all three fractions. This would be the chondroitin sulphate–protein virtually free of keratan sulphate that is enriched in the electrophoretic fractions F_{II} and F_{III}. The other polypeptide of fraction R must contain some keratan sulphate, since fraction R contained more glucosamine than did F_{II} and F_{III} (Table 2). As this polypeptide would comprise only a few per cent of the total, this may not be enough to be detected on Ouchterlony plates as a second precipitin line, since protein–polysaccharides are weak antigens. This might explain why fraction R

showed only one precipitin line although containing two polypeptide *N*-terminal amino acids.

Assuming that all protein–polysaccharides have structures consistent with the Mathews–Partridge model (Mathews & Lozaityte, 1958; Partridge *et al.* 1961), the small difference in protein content of fractions R and E (Table 3) would not alone account for their difference in size. Since the chains of chondroitin sulphate were about 28 disaccharide units in length in both fractions, their difference in size must be due mainly to a difference in the total number of carbohydrate chains in each. A smaller proportion of the total protein was bound to carbohydrate by alkali-sensitive bonds in the larger fraction E, however. Thus in fraction E, which contained 7·2% of protein, 50% of the hydroxy amino acids was destroyed by alkali, whereas 60% was destroyed in fraction R, which contained 5·3% of protein, and in a fraction containing no more than 2% of protein it was calculated that most of the serine residues were bound to chondroitin sulphate (Muir & Jacobs, 1967). Hoffman *et al.* (1967*a*) found likewise that the proportion of serine that was unstable to alkali increased with the chondroitin sulphate content of PP-L fractions. They consider (Hoffman *et al.* 1967*a*,*b*) that all their PP-L fractions had the same core protein and differed only in the number of chondroitin sulphate chains attached to it. If fractions R and E contained the same core protein, however, it would not be possible to explain the inverse relationship between size and carbohydrate content or size and proportion of alkali-sensitive hydroxy amino acids. Hoffman and co-workers (Hoffman *et al.* 1967*a*,*b*) found that in their PP-L fractions the keratan sulphate content was proportional to the protein content but that the chondroitin sulphate content was inversely related to it, in agreement with the present results. The larger size and higher proportion of keratan sulphate in fraction E than in fraction R again could not be explained if the same core protein were common to both. On the other hand, if there were several core proteins, as suggested by the presence of a number of *N*-terminal amino acids and the differences in amino acid composition, then, although maintaining a fairly constant ratio of keratan sulphate to protein, larger compounds would have longer protein cores with a greater total number of carbohydrate chains per molecule but a lower proportion of chondroitin sulphate relative to keratan sulphate. This is consistent with the finding that the larger compounds were not aggregates of smaller ones. An alternative possibility is that keratan sulphate–protein holds together varying numbers of chondroitin sulphate–proteins: for this structure, however, some bifunctional group or residue would be required.

The agreement between the two methods of calculating the length of chondroitin sulphate chains, based on the molar proportions of xylose and galactosamine and on the amount of serine destroyed during β-elimination, suggests that chondroitin sulphate was linked to serine residues alone. The threonine that was also destroyed during β-elimination may be linked to keratan sulphate, since the number of keratan sulphate chains in fractions R and E approached the number of threonine residues destroyed. The length of the keratan sulphate chains in preparation II was calculated to be 12 disaccharide units (Tsiganos, 1968), from which the number of keratan sulphate chains in the two fractions was estimated. In skeletal keratan sulphate of some other species the chains appear to be attached to both serine and threonine (Seno *et al.* 1965).

In accordance with its higher keratan sulphate content, fraction E contained more galactose than did fraction R (Table 3), but in both fractions approximately a quarter of the total galactose could not be accounted for by the galactose residues in the linkage region and that in keratan sulphate. Although the analyses for hexose were the least accurate, this rather large discrepancy suggests some other structural feature, particularly as no detectable glycoprotein was present, as shown by the absence of sialic acid, fucose and serum proteins (Tsiganos & Muir, 1969).

From the present results it appears that cartilage normally contains a heterogeneous population of protein–polysaccharides, and that some selection takes place during sequential extraction. Only the smallest compounds have been obtained with any degree of purity, but by extending the range of physical separation methods the larger compounds can perhaps be separated and their degree of heterogeneity discerned. Heterogeneity may be necessary to provide a range of physical function within a tissue. On the other hand, some of this heterogeneity might represent the catabolic state *in vivo*, so that the population of protein–polysaccharides may vary with development, maturity and aging.

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