Studies on Protein–Polysaccharides from Pig Laryngeal Cartilage

EXTRACTION AND PURIFICATION

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1. Protein–polysaccharides of chondroitin sulphate were extracted from fresh laryngeal cartilage at pH 6·8 by two procedures. Procedure I consisted of brief low-speed homogenization in 0·15 M (iso-osmotic) sodium acetate and procedure II consisted of longer homogenization followed by prolonged extraction in 10% calcium chloride solution. 2. The protein–polysaccharides in both extracts were isolated and purified by precipitation with 9-aminoacridine hydrochloride. They were free from serum proteins, collagen and nucleic acids and also of degradative enzymes. The absence of such enzymes was shown by viscosity measurements on solutions of protein–polysaccharides incubated for up to 24 hr. at pH 4 and 6·8. 3. Mannose, glucose or fucose were not detected by paper chromatography and only traces of sialic acid were present. 4. The yield with procedure II was twice that with procedure I and the products differed in their protein and glucosamine contents. 5. Hyaluronic acid was unlikely to have been precipitated at an acid pH, so the glucosamine was attributed to keratan sulphate, as serum proteins were absent. There was no free keratan sulphate in the preparation. 6. Both preparations were heterogeneous in the ultracentrifuge, showing at least three components.

Recent evidence (Mashburn, Hoffman, Anderson & Meyer, 1965; Rosenberg, Johnson & Schubert, 1965; Franek & Dunstone, 1967; Rosenberg, Schubert & Sandson, 1967; Muir & Jacobs, 1967; reviewed by Muir, 1967) suggests that chondroitin sulphate–proteins occur as a group of closely related compounds. It has been pointed out (Muir & Jacobs, 1967) that different methods of preparation may select different proportions of the compounds present in the same tissue. The present results provide some evidence for this suggestion and have been published in preliminary reports (Tsiganos & Muir, 1966; Tsiganos, 1968). In this and the following paper the term 'protein–polysaccharide' refers to compounds in which a protein moiety is covalently bound to several sulphated polysaccharides or glycosaminoglycans (Jeanloz, 1960), the latter being defined as linear polymers containing disaccharide repeating units of which one component is an amino sugar.

MATERIALS AND METHODS

All reagents were of analytical grade, including acetone and ethanol (RR grade; James Burrough Ltd., London S.E.11), with the exception of 9-aminoacridine hydrochloride, glucosamine hydrochloride, galactosamine hydrochloride, glucuronolactone, *N*-acetylneuraminic acid,

galactose, authentic neutral sugars, CPC* and acetylacetone, which was redistilled (b.p. 133–134°).

Koch–Light Laboratories Ltd., Colnbrook, Bucks., supplied bovine serum albumin, CPC, di-isopropyl phosphorofluoridate, 6-aminohexanoic acid and *p*-chloromercuribenzoate.

Analytical methods

Hexuronic acid. Hexuronic acid was determined by a modification (Bitter & Muir, 1962) of the method of Dische (1947), with glucuronolactone as a standard. Interference due to hexose was accounted for by including approx. 5% of galactose in the blanks.

Hexosamine. Samples of known weights (approx. 1 mg.) in 1 ml. of 8 M-HCl were hydrolysed under N_2 at 95° for 3 hr. (Swann & Balazs, 1966). Total hexosamine was determined as described by Muir & Jacobs (1967) by the Elson & Morgan (1933) reaction by using a modification (Bitter, 1964) of the distillation procedure of Cessi & Piliego (1960), with glucosamine hydrochloride recrystallized to constant optical rotation as a standard. Under the hydrolytic conditions used, the destruction of free glucosamine hydrochloride was only 3–4%. Corrections were made for the lower colour yield of galactosamine (Cessi & Piliego, 1960).

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

Sialic acid. Known amounts (approx. 1·5 mg.) of each preparation were hydrolysed for 1 hr. at 80° in 0·2 ml. of

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* Abbreviation: CPC, cetylpyridinium chloride.

0·05M-H_2SO_4 (Svennerholm, 1956). The sialic acid content of duplicate hydrolysates was determined by the method of Aminoff (1961). N-Acetylneuraminic acid was used as standard.

Hydroxyproline. Samples of known weight (approx. 8 mg.) of each preparation were heated in 6M-HCl for 4 hr. in an autoclave at 40 lb./in.^{2} and the hydroxyproline was determined by Stegemann's (1958) method by using an automated procedure.

Phosphate. A 7 mg. sample of each preparation was digested in H_2SO_4 and the phosphate content determined by the method of Holden & Pirie (1955), with KH_2PO_4 as a standard.

Detection of serum proteins. The Ouchterlony double-gel-diffusion technique, described in detail in the following paper (Tsiganos & Muir, 1969), was employed to detect serum proteins. Solutions of each of the preparations (5–7 mg./ml.) were allowed to diffuse against rabbit serum containing precipitating antibodies to pig serum proteins (Burroughs Wellcome and Co., London N.W.1) and the appearance of precipitin lines was observed over 1 week. Undiluted pig serum was used as a control.

Glucosamine/galactosamine molar ratios. The hexosamines were separated by ion-exchange chromatography (Gardell, 1953) by using the conditions of Crumpton (1959). To achieve the resolution needed to separate glucosamine from a large excess of galactosamine, Zeo-Karb 225 (Na$^+$ form; 8% DVB) of average particle size 15 μm. was fractionated into particles of size 10–25 μm. by the elutriation procedure of Hamilton (1958) after removal of fines by decanting four times. Batches (10–15 g.) of well hydrated resin were placed in a 2 l. pear-shaped separating funnel, and an upward flow of water of 50 ml./min. was passed through into a collecting vessel. The resin that was carried over was converted into the H$^+$ form, suspended in 0·33M-HCl and poured into a column. The packed bed volume was 40 cm. × 1·3 cm. Samples (approx. 7 mg.) of each preparation were hydrolysed in 2 ml. of 8M-HCl for 3 hr. at 95° (Swann & Balazs, 1966). After the resulting solutions were evaporated to dryness *in vacuo* over KOH pellets and P_2O_5, the residues were dissolved in water and evaporated twice more. They were then dissolved in 1 ml. of 0·33M-HCl, transferred to the column and washed in with 2 × 0·5 ml. of the acid, which was also used for elution at 12 ml./hr. Fractions (approx. 5 ml.) were collected by using a drop counter. The position of amino sugars was first determined by a direct Elson–Morgan reaction (Kraan & Muir, 1957), taking 1 ml. from each fraction. The fractions (28–31) containing glucosamine were pooled, concentrated fivefold by rotary evaporation at 40–45°, carefully neutralized (Muir & Jacobs, 1967) and then made up to 5 ml. The fractions (33–35) containing galactosamine, the principal hexosamine, were pooled, but were not concentrated before being neutralized and made up to 50 ml. The blank was prepared from fractions 26–27, which were pooled and neutralized. The hexosamine contents of the two pooled fractions were determined by the Cessi & Piliego (1960) distillation procedure.

Chromatography of neutral sugars. Descending chromatography in one dimension was performed at 18–20° with Whatman no. 1 paper and the following solvents (Smith, 1960): *A*, ethyl acetate–pyridine–water (12:5:4, by vol.) for 16 hr.; *B*, phenol–water–aq. ammonia (sp.gr. 0·88) (160:40:1, w/v/v) for 21 hr. The dried chromatograms were stained by dipping in an aniline reagent (Muir, 1958) and

heated at 95° for 7–10 min. Samples (about 7 mg.) of each preparation were hydrolysed under N_2 in 1 ml. of 1M-HCl at 100° for 3 hr. (Gregory, Laurent & Rodén, 1964) and neutralized by treatment with acidic and basic resins (Montreuil, Spik, Dumaisnil & Monsigny, 1965). The hydrolysates were diluted to 0·1M-HCl with water before being applied to a column of Dowex 50W (H$^+$ form) containing 15 ml. of resin. The column was washed with about 20 ml. of distilled water and to the effluents Dowex 3 (CO$_3^{2-}$ form) (Lindahl & Rodén, 1965) was added until the evolution of CO_2 ceased. The slurry was poured into a column containing 3 ml. of Dowex 3 (CO$_3^{2-}$ form) connected directly to a small 5 ml. column of Dowex 50W (H$^+$ form). The columns were washed with 20 ml. of distilled water, and the effluents brought to pH 6 with Dowex 3 (CO$_3^{2-}$ form) and dried by rotary evaporation at 45–50°. The residue was dissolved in about 40 μl. of water and 10 μl. applied to the paper alongside samples containing 10–15 μg. of authentic sugars.

Optical rotation. A Hilger–Watts micro-optic photoelectric polarimeter was used to measure the optical rotation of sodium salts of solutions of protein–polysaccharides in water.

Infrared spectra. A Unicam SP.200 recording spectrophotometer was used to obtain i.r. spectra. Samples (1 mg.) of dried material were mixed with 100 mg. of KBr in a ball mill and the resulting powder was pressed into a disc.

Preparation of protein–polysaccharides

The larynges of 6–8-month-old pigs, obtained within a few minutes of death while still warm, were frozen in solid CO_2. All subsequent operations were performed in a cold-room at approx. 4°. The tissue was thawed sequentially and the cartilage dissected and sliced.

Procedure I: brief extraction in 0·15M-sodium acetate at pH 6·8. A 250 g. portion of sliced cartilage was suspended in 600 ml. of sodium acetate solution cooled to 2°. Batches (approx. 200 ml.) were homogenized in a Waring Blendor operated at full speed five times for 1 min. at short intervals during which the container of the homogenizer was cooled in a acetone–solid CO_2 mixture. The temperature of the homogenate at no time rose above 4°. The cooled homogenates were quickly filtered twice through a double layer of lint and immediately to the clear filtrate a solution of 9-aminoacridine hydrochloride, saturated at 60°, was added until the precipitate flocculated, when no more precipitate formed. The time from the start of homogenization was no more than 30 min. After 2 hr. the precipitate was collected by filtration through Whatman no. 541 paper and washed with a dilute solution of 9-aminoacridine. The sodium salt of the polyanion was obtained by exchange with Zeo-Karb 225 (Na$^+$ form; 200 mesh; 8% DVB), which took up the 9-aminoacridine. A total of about 300 ml. of wet resin was used suspended in 300 ml. of water containing 10 ml. of a solution of sodium acetate, consisting of 30 g. of anhydrous sodium acetate and 15 ml. of acetic acid made up to 100 ml. with water. The suspension was shaken in a mechanical roller overnight at room temperature, by which time the precipitate had dissolved and the supernatant solution had become colourless. The resin was removed by filtration through Whatman no. 541 paper, and washed four times with 50 ml. of water until the washings no longer contained any material that was precipitated with 9-aminoacridine. The combined filtrate and washings were adjusted to pH 3·5

with acetic acid and the slightly cloudy solution was filtered through a bed of Filter-Cel about 0·5 cm. thick.

The precipitation with 9-aminoacridine was repeated once, and after regeneration of the sodium salt as before the filtrate and resin washings were filtered through a sintered-glass funnel of no. 4 porosity before concentration to 200 ml. by rotary evaporation at 45°. Then 4 vol. of cold ethanol was added, followed by a few millilitres of the above mixture of sodium acetate and acetic acid until a flocculent precipitate formed. After 15 hr. at 4° the precipitate was centrifuged and washed by resuspension and centrifuging twice in aq. 80% ethanol, twice in ethanol and once in acetone. It was dried over P_2O_5 *in vacuo*. The yield was 2·7 g.

The filtrate from the first precipitation with 9-amino-acridine was concentrated, and after removal of the acridine with Zeo-Karb 225 only traces of uronic acid were detected.

Procedure II: prolonged extraction with 10% (w/v) calcium chloride at pH 6·8. The method described by Muir & Jacobs (1967) was used, the homogenization proceeding intermittently for a total time of 20 min. at 4°. After 48 hr. at 4°, the homogenate was filtered through lint and then dialysed against water at 4°. The protein–polysaccharides were isolated as described in procedure I. The yield was about twice that by procedure I.

A sample (1 g.) of the protein–polysaccharide was further purified by dissolving in 100 ml. of 0·4 M-NaCl and addition with stirring of 11 ml. of a 10% (w/v) solution of CPC in 0·4 M-NaCl (Scott, 1960). After several hours at 37°, the precipitate was centrifuged at 3500 rev./min. for 45 min. at 20°, and washed by resuspension and centrifuging twice with 20 ml. of 1% (w/v) CPC (containing no salt) and once with 20 ml. of 0·4 M-NaCl containing 0·1% CPC. It was then dissolved in 50 ml. of 2·6 M-NaCl and the protein–polysaccharides were precipitated with 4 vol. of cold ethanol. The precipitate was centrifuged, redissolved in 50 ml. of 0·1 M-NaCl and reprecipitated with cold ethanol, and then washed and dried as described above.

The supernatant and washings from the precipitation with CPC were concentrated to about one-third the original volume. On addition of 4 vol. of cold ethanol no precipitate formed.

Viscometry

Solutions (approx. 0·5%, w/v) of the preparation obtained by procedure II in either 0·1 M-potassium acetate buffer, pH 4·0, or 0·1 M-potassium phosphate buffer, pH 7·2, were stirred for 1 hr. at 4°. They were then passed through no. 4 sintered-glass filters and 3 ml. portions were put in Ostwald capillary viscometers at $37 \pm 0.05°$. Flow times were measured after 5 min. and 1, 2, 3, 16 and 17 hr. Di-isopropyl phosphorofluoridate (1 M in propan-2-ol) and 6-amino-hexanoic acid were separately added to portions of the protein–polysaccharide in acetate buffer to give concentrations of 15 mM and 400 mM respectively; p-chloromercuri-benzoate (10 mM in 0·1 M-NaHCO₃, pH 8·6) was added to the corresponding solution of protein–polysaccharide in phosphate buffer, to give a concentration of 0·2 mM. The flow times of these solutions were measured at the same times as above.

In a separate experiment, 50 mg. of protein–polysaccharide from procedure II was dissolved in 20 ml. of acetate buffer, pH 4·0, and incubated at 37° under toluene for 24 hr. It was then precipitated with 4 vol. of cold ethanol, centri-

fuged, washed and dried as described above. Solutions of the same concentration, approx. 0·5% (w/v) of the original (procedure II) and precipitated materials were prepared in acetate buffer, and the flow times were measured at similar times.

Stock solutions of exactly 2% of each of the original and reprecipitated protein–polysaccharides from procedure II were prepared in 4 M-guanidine hydrochloride, pH 4·4, filtered and diluted with the same solvent to the same three concentrations. Flow times of each series of dilutions were measured in the same viscometer at 37°. Intrinsic viscosities were determined from plots of reduced viscosity versus concentration.

Bacterial growth was not detected in any of the solutions at the end of the experiments.

RESULTS AND DISCUSSION

The products of each method of preparation differed particularly in the molar ratio of glucosamine to galactosamine and to a smaller extent in protein content. Brief extraction with neutral iso-osmotic sodium acetate solution (procedure I) gave a product with the following analysis: Found: hexosamine, 29·5; hexuronic acid, 27·5; protein, 7%; molar ratio of glucosamine to galactosamine 1:26; $[\alpha]_D^{22} -33 \pm 1°$ (c 1·002 in water). The optical rotation of the product of prolonged extraction with neutral hyperosmotic calcium chloride (procedure II) was similar, $[\alpha]_D^{22} -29 \pm 1°$, but in contrast the product contained almost twice the relative proportion of glucosamine, since the molar ratio of glucosamine to galactosamine was 1:14. In addition, it contained somewhat more protein, namely 10%.

The i.r. spectra, however, were indistinguishable, with prominent bands at 920 and 855 cm.⁻¹ characteristic of chondroitin 4-sulphate but not that at 820 cm.⁻¹ characteristic of chondroitin 6-sulphate (Hoffman, Linker & Meyer, 1958; Mathews, 1958). Neither the i.r. spectrum nor the optical rotation would completely exclude the presence of some chondroitin 6-sulphate. However, when the glycosaminoglycans obtained from whole cartilage after digestion with papain were fractionated by the CPC method (Antonopoulos & Gardell, 1963), the fraction that should have contained chondroitin 6-sulphate showed the i.r. spectrum of the 4-isomer (C. P. Tsiganos, unpublished work). On the other hand Mathews & Glagov (1966) found that about half the chondroitin sulphate of human thyroid and cricoid cartilage consisted of the 6-isomer; species differences have been suggested by them.

On high-speed homogenization in water (Malawista & Schubert, 1958; Gerber, Franklin & Schubert, 1960) cartilage is completely disintegrated to give yields of 5–15% of a product known as protein–polysaccharide light fraction or PP-L. In contrast, by using the procedures described here, the cartilage was only partially disintegrated,

although disintegration was more extensive in procedure II, where the yield of protein–polysaccharide was about 2%. Such differences in yield and the difference in composition between each product suggests that different molecular species are not extracted with equal ease. The higher yields with procedure II may be due in part to the lyotropic effect of calcium chloride on collagen, so that protein–polysaccharides more closely associated with collagen may be extracted in this procedure.

The glucosamine present in each product could not be accounted for by contamination with glycoproteins for the following reasons: serum proteins were not detected by immunological methods and, although xylose and galactose were found, these sugars are attributable to the atypical sequence of sugars linking chondroitin sulphate chains to the protein core (Gregory et al. 1964; Lindahl & Rodén, 1966; Rodén & Armand, 1966). Further, even though the conditions used to hydrolyse and isolate neutral sugars were designed to minimize losses (Montreuil et al. 1965; Neuberger & Marshall, 1966; Gottschalk, 1966), mannose, glucose or fucose were not detected and only traces of sialic acid were found. The absence of the last two sugars may imply a species difference, since they have been identified in protein–polysaccharides from bovine cartilage (Gregory et al. 1964; Luscombe & Phelps, 1967), although glycoproteins have also been shown to be present in some PP-L preparations (Dunstone & Franek, 1967). Collagen or nucleic acid was not present in either of the preparations, since hydroxyproline and phosphate were scarcely detectable.

Hyaluronic acid was unlikely to be present, because the protein–polysaccharides were precipitated at pH 3·5 (Bitter & Muir, 1966), and hence the glucosamine could be attributed to keratan sulphate, which has been shown to be an integral part of chondroitin sulphate–proteins (Gregory et al. 1964; Heinegård & Gardell, 1967; Tsiganos & Muir, 1967). Keratan sulphate itself, freed of protein and chondroitin sulphate, is soluble in excess of CPC in the absence of salt, whereas hyaluronic acid is retained in solution in the presence of 0·4 M-sodium chloride (Scott, 1960; Antonopoulos, Borelius, Gardell, Hamnström & Scott, 1961). Attempts to remove keratan sulphate and hyaluronic acid from the chondroitin sulphate–protein by the CPC method failed to decrease the proportion of glucosamine or to produce material in the supernatant that would be precipitated with ethanol. Keratan sulphate would thus appear to be part of these macromolecules, the proportion being lowest in those that were more easily extracted and contained less protein.

Preparations of bovine PP-L obtained by complete homogenization of cartilage in water followed by precipitation with ethanol contain proteolytic enzymes that have both neutral and acidic pH optima (Partridge, Whiting & Davis, 1965; Dziewiatkowski, Tourtellotte & Campo, 1967; Serafini-Fracassini, Peters & Floreani, 1967). These are removed if the PP-L is reprecipitated with cationic reagents (Dziewiatkowski et al. 1967; Serafini-Fracassini et al. 1967). Lysosomal proteases, which have an acidic pH optimum, are released by water from cartilage (Lucy, Dingle & Fell, 1961). The protein–polysaccharide obtained by procedure I with neutral iso-osmotic sodium acetate is therefore less prone to degradative enzymes than is PP-L. The absence of uronic acid in the supernatant from the precipitation with 9-aminoacridine showed that at least there were no fragments too small to be precipitated by this reagent.

Owing to the probable shape of chondroitin sulphate–proteins (Mathews & Lozaityte, 1958;

Table 1. *Percentage in relative viscosity during incubation at 37° in 0·1 M-phosphate buffer, pH 7·2, and in 0·1 M-acetate buffer, pH 4·0, of the original chondroitin sulphate–protein obtained by procedure II and of the reprecipitated material*

For further details, see the text. Abbreviations: pCMB, p-chloromercuribenzoate; DFP, di-isopropyl phosphorofluoridate; 6-AHA, 6-aminohexanoic acid.

	Original material						Reprecipitated material pH 4·0
	pH 7·2		pH 4·0				
Inhibitor	None	pCMB (0·2 mM)	None	DFP (7·0 mM)	DFP (15 mM)	6-AHA (400 mM)	None
Incubation time (hr.)							
0 (5 min.)	0	0	0	0	0	0	0
1	10	9	7	7	8	6	5
2	15	15	13	13	12	12	12
3	17	18	17	17	16	15	15
16	34	33	30	30	—	—	25
17	36	34	31	32	—	28	28

Partridge, Davis & Adair, 1961), a large decrease in viscosity follows the scission of only a very few peptide bonds (Mathews, 1956; Muir, 1958; Cessi & Bernardi, 1965; Dziewiatkowski et al. 1967) and hence the presence of any protease should be revealed most easily by viscometry. After a rapid decrease in viscosity over the first 2hr., a constant value was reached after 16–17hr. when the product of procedure II was incubated at 37° in buffers at either pH 4·0 or pH 7·2, and the addition of three enzyme inhibitors had no effect (Table 1). It appears that the fall in viscosity was due to some molecular rearrangement, since the original viscosity was regained when the protein–polysaccharides were reprecipitated with ethanol, dried and redissolved, after which the same fall in viscosity was observed. A similar decrease in viscosity was observed by Malawista & Schubert (1958) with bovine PP-L. In 4M-guanidine hydrochloride at pH 4·4, however, there was no change in viscosity over many hours, presumably because it reduces interactions between the highly charged molecules; 4M-guanidine hydrochloride was therefore employed when intrinsic viscosities of the original and reprecipitated materials were determined. No difference was found.

Sedimentation-velocity experiments in 4M-guanidine hydrochloride revealed that both preparations were heterogeneous. The slowest-moving component in preparation I consistituted about half the total (Fig. 1); the heavier, however, separated into two components.

The results suggest that the differences in composition between the two preparations were unlikely to have arisen from the effects of degradative enzymes and therefore that the method of extraction determines what molecular species are obtained. In the following paper (Tsiganos & Muir, 1969) the separation by gel filtration and characterization of the smallest protein–polysaccharides is described.
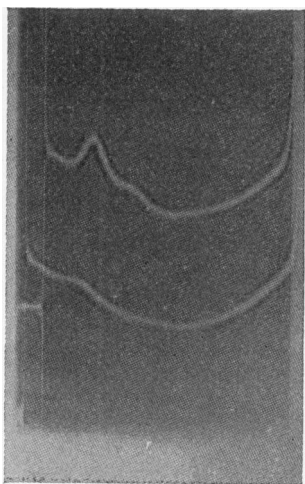
Fig. 1. Sedimentation-velocity patterns of preparation I in 4M-guanidine hydrochloride–0·02M-phosphate buffer, pH 6·5, at 25°; top pattern, 6mg./ml.; bottom pattern, 3mg./ml. The photograph was taken after 112min., at 59 780rev./min. with bar angle 60°. Sedimentation proceeds from left to right and was performed in a Beckman Spinco model E analytical ultracentrifuge.

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