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Protein–Polysaccharides of Pig Laryngeal Cartilage

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1. Protein–polysaccharides of chondroitin 4-sulphate were extracted with neutral calcium chloride from pig laryngeal cartilage that was not completely homogenized. The protein–polysaccharides were purified by precipitation with 9-aminoacridine. On zone electrophoresis in compressed glass fibre at pH 7·2 it was separated into two fractions, although two distinct zones were not obtained. These fractions, which had already been shown to differ in their antigenic determinants, also differed considerably in amino acid composition, total protein, hexose and glucosamine contents. 2. The fraction of higher mobility contained approx. 2% of protein and only traces of glucosamine. Serine and glycine accounted for over half the total amino acid residues, but aromatic, basic and sulphur-containing amino acids were not detected. The weight-average molecular weight, determined by sedimentation, was 230000. 3. Assuming that there was the same sequence of neutral sugars at the linkage points as in PP-L fraction (protein–polysaccharide light fraction), the approximate molar ratio of hexose to serine suggested that most of the serine residues were linked to chondroitin sulphate chains. Support for this was derived from the agreement between the weight-average molecular weight of the chondroitin sulphate–peptide after proteolysis, and the chain weight calculated from its serine content. The chain weight based on the serine content of the fraction of higher electrophoretic mobility was approximately similar. 4. In contrast, the fraction of lower electrophoretic mobility resembled PP-L fraction in its amino acid composition, protein and glucosamine contents. The presence of glucosamine, together with the higher hexose content, suggested that this fraction contained some keratan sulphate. 5. The relatively low molecular weight of the fraction of higher mobility enabled it to be extracted without complete disintegration of the cartilage. The unlikelihood of its being produced by autolytic enzymes is discussed.

Chondroitin sulphate in cartilage is combined with non-collagenous protein (Malawista & Schubert, 1958; Muir, 1958; Partridge & Davis, 1958; Partridge & Elsden, 1961) through a covalent bond to serine (Muir, 1958; Gregory, Laurent & Rodén, 1964). Since alkali cleaves this bond (Muir, 1958) by a β-elimination reaction (Anderson, Hoffman & Meyer, 1965), the hydroxyl group of serine is involved. Different protein–polysaccharide preparations, however, do not have the same amino acid composition, which may in part be due to species or tissue differences. Nevertheless preparations differing in amino acid composition have been obtained even from the same tissue such as bovine nasal septum (Scheinthal & Schubert, 1963; Gregory *et al.* 1964; Anderson *et al.* 1965) by essentially the same procedure in which disintegrated cartilage was extracted with water (Gerber, Franklin & Schubert, 1960). This suggests that a variety of protein–polysaccharides may exist in the same tissue, different preparations selecting different proportions of those present. The results of the present work support this view. Protein–polysaccharides from pig laryngeal cartilage were separated into two fractions by electrophoresis. They differed in protein content, amino acid composition and antigenic determinants.

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MATERIALS AND METHODS

With the exception of 9-aminoacridine hydrochloride, glucuronolactone, glucosamine hydrochloride and galactose, the reagents were all of analytical grade including acetone and ethanol (R.R. grade; James Burroughs Ltd., London, S.E. 11).

Hexosamine. A known weight of material (5–10mg.) was dissolved in 5ml. of water. Samples (1ml.) were placed in

tubes and evaporated to dryness *in vacuo*. To each, 1ml. of 4N-HCl was added and the air displaced with nitrogen, and the tubes were sealed and placed at once in a boiling-water bath. After 8hr. the tubes were cooled and the contents washed out quantitatively into 20ml. or 25ml. volumetric flasks cooled in ice–water. A drop of phenolphthalein was added and the acid neutralized with 2N-NaOH. On reaching the end point 1 drop of N-HCl was added, before making up to volume. Samples (1ml.) of the solutions, which contained approx. 10–25μg. of hexosamine, were taken and the hexosamine was estimated by the Elson & Morgan (1933) reaction, by using a modification of the distillation procedure of Cessi & Piliego (1960), in which the reagents were as follows: A, 4ml. of redistilled acetylacetone dissolved in 100ml. of 0·5M-Na_2CO_3, final pH10·0; B, 1g. of dimethylaminobenzaldehyde dissolved in 100ml. of ethanol containing 5ml. of conc. HCl. Then 1ml. of the sample or standard was heated with 3ml. of reagent A in a vigorously-boiling-water bath for 23min. The chromogen was then distilled rapidly into 5ml. volumetric flasks containing 3·5ml. of reagent B until the 5ml. mark was reached. After 45min. in the dark at room temperature the extinction was read at 545mμ. Glucosamine hydrochloride recrystallized to constant rotation was used as the standard.

Hexose. The method of Trevelyan & Harrison (1952) was used, except that, to compensate for interference by the large amounts of uronic acid and hexosamine in the samples, approximately similar amounts were added to the control tubes as an equimolar mixture of glucuronolactone and glucosamine. Galactose was used as a standard.

Sulphur. The total sulphur was determined after combustion by Pascher und Pascher, Buchstrasse 54, Bonn, Germany.

Sulphate. Samples (2ml.) of an aqueous solution containing a known weight (about 3mg./ml.) of material were placed in tubes and 2ml. of 60% (v/v) formic acid was added to each. A control was also prepared consisting of 2ml. of water and 2ml. of 60% formic acid. The tubes were sealed and heated at 100° for 8hr. On cooling the contents were washed out quantitatively into 10ml. volumetric flasks and made up to volume. Samples (1ml.) of the control and of the hydrolysates containing approx. 60–100μg. of sulphate (as SO_4^{2-})/ml. were taken and the sulphate content was estimated by a modification (R. Ewins & H. Muir, unpublished work) of the method of Jones & Letham (1954), the control sample being used as the blank. Potassium sulphate dried to constant weight at 80° *in vacuo* over P_2O_5 was used as a standard.

Uronic acid. Uronic acid was estimated by a modification (Bitter & Muir, 1962) of the method of Dische (1947), glucuronolacetone being used as a standard.

Amino acid. A known weight of about 30mg. of material was dissolved in 25ml. of deionized water (Jacobs, 1955). Three 1ml. samples were first removed for the estimation of nitrogen as described below. The remainder was transferred to a Pyrex bottle, brought to 6N-HCl with concentrated acid and the air displaced with a stream of nitrogen before the container was sealed. It was heated at 105° for 24hr. The hydrolysate was filtered through Whatman no. 50 paper, which was washed several times with deionized water. The combined filtrate and washings were evaporated to dryness *in vacuo* over NaOH at room temperature. The residue was dissolved in 10ml. of sodium citrate buffer, pH2·2, containing 2% (v/v) of thiodiglycol. Samples (2ml.) were used to obtain duplicate amino acid analyses by an improved (Jacobs, 1964) two-column method with the Beckman–Spinco model 120 amino acid analyser. The ratio of glucosamine to galactosamine could thus be determined at the same time as the basic amino acids, even when either sugar was present only in traces. Under the conditions used to hydrolyse amino acids, however, losses of amino sugars were 33–46%, most of the nitrogen being recovered as ammonia.

Total nitrogen. Three 1ml. samples of both the original aqueous solution of protein–polysaccharide and the hydrolysed sample in citrate buffer were placed in 5ml. Pyrex glass ampoules and evaporated to dryness. Conc. H_2SO_4 (0·2ml.) was added to each sample and the ampoules were sealed. Their nitrogen content was determined by the indanetrione hydrate method (Jacobs, 1962). Nitrogen was also determined by the Dumas method by Pascher und Pascher of Bonn.

Absorption spectra. A Unicam SP.200 recording spectrophotometer was used to obtain infrared absorption spectra. Samples (1mg.) of material were mixed with 100mg. of KCl in a ball mill and the resulting powder was pressed into a disk.

A Unicam SP.700 recording spectrophotometer was used to obtain ultraviolet absorption spectra of solutions of 0·4–0·1% (w/v) of protein–polysaccharides in 0·17M-NaCl.

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

Optical-rotatory-dispersion curves were kindly determined by Professor W. Klyne and Dr Mary Coles with protein–polysaccharide solutions in 0·15M-KCl.

Estimation of molecular weights. Dr. R. H. Pain kindly estimated molecular weights by the sedimentation-equilibrium method of Yphantis (1960) with a Spinco model E ultracentrifuge. The buffer was 0·5M-KCl–glycine pH3·0. A partial specific volume of 0·57 (Tanford, Marler, Jury & Davidson, 1964) was used in the calculations.

Extraction of protein–polysaccharide. The larynges of 6–8-month-old pigs were obtained within a few minutes of death and while still warm were frozen in solid CO_2. All subsequent operations were performed in a cold room at approx. 4°. The protein–polysaccharide was prepared essentially as described by Muir (1958) apart from the following changes. Only the thyroid and cricoid cartilages were used. The larynges were thawed sequentially and dissected, and the cartilage was sliced and placed at once in cold 10% (w/v) $CaCl_2$ adjusted to pH6·8 with 2N-NaOH. A total of 1·3kg. of sliced cartilage was first soaked for 24hr. in 4l. of 10% $CaCl_2$. Batches (approx. 200ml.) of the suspension were homogenized for a few minutes at a time so that the temperature did not rise above 10°. The homogenate, which was not completely disintegrated, was kept for 48hr. at 4° before it was filtered through a bed approx. 2·5cm. thick made of Celite 545–Celite 535 (3:1, w/w) on top of two pieces of Whatman no. 541 filter paper supported by two layers of lint. The clear filtrate was dialysed against two changes of 30l. of distilled water at 4° and no ammonium sulphate was added as was done previously. The cloudy precipitate that formed during the dialysis dissolved partially when the solution was adjusted to pH6·5 with

0·4M-sodium acetate. The precipitate that remained was removed by centrifuging.

The first precipitation of the protein–polysaccharide as done previously with $Co(NH_3)_6Cl_3$ was omitted and replaced by two precipitations with 9-aminoacridine hydrochloride, the first from a volume of about 5l. and the second from 1·5l. The sodium salt of the polyanion was regenerated as before by exchange with the Na^+ form of Zeo-Karb 225 (mesh 8% DVB) which took up the aminoacridine. The solution of the sodium salt regenerated after the first precipitation was adjusted to pH 6 with 0·5M-$NaHCO_3$ and filtered through Celite 503 on Whatman no. 42 paper. After the second precipitation, the regenerated sodium salt and resin washings were filtered through Whatman no. 42 paper and the filtrate was concentrated to approx. 200ml. Then 4vol. of ethanol was added with stirring, followed by 70ml. of ethanol saturated with sodium acetate. After standing for 48hr. at 4°, the precipitate was centrifuged and washed by resuspending and centrifuging twice in aq. 80% (v/v) ethanol, twice in ethanol and once in acetone. This washing procedure was used whenever a sodium salt was isolated. It was finally dried over P_2O_5 *in vacuo*.

Preparative electrophoresis in compressed glass fibre. The apparatus containing the fibre bed was constructed as follows (obtainable from Wright Scientific Ltd., London, N.W. 6). To obtain even cooling above and below the trough containing the fibre, holes were bored in the solid brass base and lid through which water was circulated from a large reservoir at 4°. The base was electrically insulated by a sheet of Perspex $\frac{1}{32}$in. thick and the lid by a sheet of polyvinyl chloride–polyvinyl acetate copolymer (heavy grade). The trough, 25·2cm.×9·6cm., had Perspex sides and ends 3·8cm. deep and 2·5cm. thick, screwed to the base through a rubber gasket. The buffer reservoirs communicated with the trough through four oval holes 2·5cm.×1·3cm., placed 1·3cm. apart, cut through the Perspex at each end. The buffer reservoirs were made of Perspex 15cm.×6·3cm.×7·5cm. divided in half by a Perspex sheet with communicating holes. The reservoirs contained approx. 500ml. and the electrodes of platinum wire were placed in the outer compartment.

Four sheets of Whatman GF/B glass paper were homogenized to a thick slurry in 0·1M-phosphate buffer, pH 7·2. About half the slurry was spread evenly in the trough. A piece of Perspex 25cm.×9·4cm. and block of wood of the same area were placed in the trough, and the glass-fibre slurry was compressed with a screw press until the pressure reached approx. 500lb./in.^{2} (measured with a torque spanner), while the buffer squeezed out was sucked off. The remainder of the slurry was applied and compressed, the glass-fibre block being then approx. 2·5cm. thick. A slot 3mm.×4cm. was cut across the middle of the block. Approx. 200–400mg. of protein–polysaccharide dissolved overnight in 2–4ml. of buffer was injected into the slot with a syringe. After 10min. the brass lid was put on and screwed down with four wing-nuts until there was slight extrusion of the buffer. The buffer reservoirs were then filled until the level reached the top of the glass-fibre block. A potential difference of 15–20v/cm. was applied for 2½–3½hr., the contents of the reservoirs being interchanged twice. The temperature of the block did not rise above 10°. The position of the protein–polysaccharide was determined by pressing dry filter-paper strips on to the block until moist, drying and staining with aq. 0·1% toluidine blue. Before the pressure on the block was released, however, the buffer was withdrawn from the reservoirs because glass fibre imbibes a lot of water very quickly. The band of protein–polysaccharide, which at the start was about 0·5cm. wide, spread towards the anode to a width of 4·5–5cm., the edge nearest the cathode remaining near the origin. The half of the band nearer the anode was cut out and dispersed in 1l. of water. The remainder of the band was treated likewise. After stirring for 2hr., the glass fibre was removed on sintered-glass funnels of porosity grade 1 and washed twice by resuspending in 1l. of water and filtering. Filtrate and washings were combined, concentrated to approx. 500ml. by rotary evaporation and dialysed against two changes of 5l. of distilled water. The solutions were finally concentrated to 10–15ml. and filtered through grade 3 sintered-glass funnels, and the protein–polysaccharides were precipitated with 6vol. of ethanol to which was added dropwise a mixture containing 2·5M-sodium acetate and acetic acid until the precipitates flocculated. After standing for 24hr. the precipitates were collected and washed with ethanol and acetone and dried as described above. The faster-moving fractions were combined and the electrophoresis was repeated twice with approx. 150mg. of material. The faster-moving fraction from the third electrophoresis was desalted by passing through a column (145cm.×3·5cm.) of Sephadex G-25 eluted with 0·1N-acetic acid. The protein–polysaccharide in the void volume was concentrated and precipitated as described above. The slower-moving fraction from the first electrophoresis was desalted likewise. Before analysis all samples were dried to constant weight at 80° *in vacuo* over P_2O_5.

Chondroitin sulphate–peptide. A 400mg. sample of unfractionated protein–polysaccharide in 80ml. of 0·1M-borate buffer, pH 8·2, containing 0·02M-$CaCl_2$, was treated with 60mg. of Pronase from *Streptomyces griseus* (Nomoto & Narahashi, 1959). It was incubated for 40hr. at 37° under toluene. The solution was diluted with an equal volume of water and the polysaccharide precipitated with 9-aminoacridine. After regenerating the sodium salt it was further digested with activated papain (Muir, 1958). The polysaccharide was again precipitated with 9-aminoacridine and finally isolated as the sodium salt as described for the protein–polysaccharide. It was then subjected in the same way to electrophoresis in glass fibre, when it travelled as a compact band towards the anode. It was recovered from the glass fibre and passed through Sephadex G-25, before the sodium salt was precipitated, washed and dried [Found: hexosamine, 33·0; hexuronic acid, 30·5; sulphate, 14·1; N, 3·01%. Serine 54·5±5·6μmoles/g. (mean of five chromatographic determinations, corrected for 13% destruction of serine during hydrolysis)].

Alkali treatment of the chondroitin sulphate–peptide. A 70mg. sample of the peptide was dissolved in 2ml. of 0·5N-NaOH. After 24hr. at room temperature it was neutralized with N-HCl and dialysed overnight. The polysaccharide was precipitated with 9-aminoacridine and the regenerated sodium salt passed through Sephadex G-25 before it was precipitated, washed and dried (Found: N, 2·74%).

Dinitrophenylation. A 48mg. sample of the faster electrophoretic fraction was dinitrophenylated by the procedure of Durant, Hendrickson & Montgomery (1962), except

that when all the reagent had been added the solution was kept overnight at room temperature in the dark. After extraction with ether, the solution was dialysed, concentrated under reduced pressure to about 5ml. before the sodium salt was precipitated and washed in the usual way with ethanol and acetone. It was again dissolved in water, precipitated and washed. The yield was 35mg.

A 82mg. sample of chondroitin sulphate peptide was dinitrophenylated in the same way. The yield was 69mg.

The extinctions of solutions of each compound in water were measured at 355mμ.

Procedures described by Fraenkel-Conrat, Harris & Levy (1955) were used to examine DNP-amino acids liberated on hydrolysis.

RESULTS

The infrared spectra of the two electrophoretic fractions were virtually identical and showed that they contained chondroitin 4-sulphate, since there were bands at 920 and 855cm.$^{-1}$ and none at 820cm.$^{-1}$ characteristic of chondroitin 6-sulphate (Hoffman, Linker & Meyer, 1958; Mathews, 1958; Lloyd & Dodgson, 1959). Their optical rotations were also consistent with that recorded for chondroitin 4-sulphate (Meyer, Davidson, Linker & Hoffman, 1956), the fraction of higher mobility having a slightly greater negative rotation than that of lower mobility.

The negative optical rotatory dispersion of the faster electrophoretic fraction increased continuously without inflexions from 300 to 210mμ, as did that of the slower-running fraction from 400 to 220mμ. The dispersions could not be measured below these wavelengths because the solutions became too opalescent.

Despite these resemblances, however, differences in carbohydrate constituents, nitrogen and sulphate contents were found. The analyses were as follows:

Electrophoretic fraction of higher mobility [Found: hexosamine, 34·5; hexuronic acid, 29·5; hexose, 1·7; sulphate, 14·6; S, 5·2; N, 3·10 (by Kjeldahl; Jacobs, 1962), 3·14 (by Dumas) %], $[\alpha]_D^{21} - 35{\cdot}5 \pm 1°$ (*c* 1·006 in water).

Electrophoretic fraction of lower mobility (Found: hexosamine, 26·8; hexuronic acid, 27·8; hexose, 6·0; sulphate, 13·0; N, 4·82%), $[\alpha]_D^{21} - 30{\cdot}8 \pm 0{\cdot}8°$ (*c* 1·008 in water).

A particularly striking difference was that the faster-moving fraction contained almost no glucosamine, since the molar ratio of galactosamine to glucosamine was 133:1, whereas in the slower-moving fraction it was 7·76:1, although their total hexosamine contents were not grossly different.

These ratios were derived from the values in Table 1 determined at the same time as amino acids after hydrolysis in 6N-hydrochloric acid for 24hr. when at least one-third of the amino sugars were destroyed. The values for amino sugars in Table 1 are thus considerably below the values for total hexosamine determined after hydrolysis for 8hr. in 4N-hydrochloric acid. The ratios of amino sugars are therefore only approximate but sufficiently different to be significant.

Table 1. *Amino acid composition of electrophoretic fractions of protein–polysaccharides from pig laryngeal cartilage*

Duplicate analyses are expressed as μmoles/g. of protein–polysaccharide. Samples were hydrolysed in 6N-HCl at 105° for 24hr. The values for serine and glycine alone have been corrected for losses during hydrolysis. $CySO_3H$, Cysteic acid; MetSO, methionine sulphoxide.

	Fraction of higher mobility		Fraction of lower mobility	
Asp	9·6	10·1	85·4	85·6
Thr	2·9	3·3	62·4	62·5
Ser	58·8	61·2	116·4	115·0
Glu	15·9	16·4	126·0	124·2
Pro	20·5	16·6	72·9	73·1
Gly	41·8	42·2	128·3	120·8
Ala	10·9	11·2	100·7	97·8
$CySo_3H$	—	—	6·2	6·1
Val	6·2	6·2	69·9	70·5
MetSO	—	—	16·1	10·3
Ile	2·0	2·4	42·6	42·9
Leu	10·2	10·9	88·2	86·8
Tyr	—	—	7·6	8·0
Phe	—	—	33·3	34·9
Lys	—	—	36·2	36·1
His	—	—	15·8	15·7
Arg	—	—	43·2	42·6
Glucosamine*	9·1	9·2	102·9	103·5
Galactosamine*	1203·0	1260·0	791·0	810·0
Ammonia	615·0	636·0	462·0	462·5

* Not corrected for considerable losses during hydrolysis in 6N-HCl.

The amino acid composition and content of each fraction was also considerably different (Table 1). The protein or polypeptide in the faster-moving fraction amounted to approx. 2% of the total weight when estimated either by a summation of the amino acid analyses or by the difference between total nitrogen and hexosamine nitrogen. There was no chromatographic or spectroscopic evidence that aromatic amino acids were present, nor were basic amino acids detectable by chromatographic analysis, making the amino acid composition of this fraction unusually simple. Serine accounted for approximately one-third and glycine for one-quarter of the total amino acid residues (Table 1).

In contrast, the electrophoretic fraction of lower mobility contained approx. 10% of protein, which had a more complex amino acid composition. Aromatic, basic and sulphur-containing amino

acids were present, and glutamic acid rather than serine predominated, followed by glycine and serine (Table 1).

Other less-marked differences were that the fraction of higher mobility contained somewhat more hexuronic acid and sulphate and significantly less hexose than the fraction of lower mobility. Although the hexose analyses were the least accurate, the difference between the two fractions was too large to be fortuitous.

Dinitrophenylation indicated that there was approximately one available amino group in a weight of 47500 in the fraction of higher mobility, assuming from the amino acid composition shown in Table 1 an average molecular extinction of 15500 (Rao & Sober, 1960) for any amino acids that may have reacted with fluorodinitrobenzene (preliminary attempts to identify *N*-terminal amino acids were not successful). In contrast, the weight-average molecular weight determined by sedimentation was 230000, although it was rather polydisperse.

Dinitrophenylation of the chondroitin sulphate–peptide did not reveal any single *N*-terminal amino acid. After hydrolysis several DNP-amino acids were identified, with a preponderance of serine and glycine. An average molecular extinction of 15800 and 15400 was assumed for the mixture of statistically possible DNP-amino acids (Rao & Sober, 1960), based on the amino acid composition of two preparations of the peptide. From these values and the extinction of the DNP derivative of the chondroitin sulphate–peptide, its chain weight was calculated to be 19500 and 19000 respectively. The weight-average molecular weight determined by sedimentation was 19200. The extinctions recorded by Rao & Sober (1960) were measured in N-sodium hydroxide. Since the extinctions of a preparation of the DNP-peptide or DNP-amino acids increased by 20% in N-sodium hydroxide compared with water, all extinctions measured in water were increased by 20% for use in the calculations.

The serine content of the protein–polysaccharide of higher electrophoretic mobility was 60 μmoles/g., and that of the chondroitin sulphate–peptide was 54·5 μmoles/g., indicating that little serine was lost on proteolysis. Assuming that most of the serine residues were therefore attached to chondroitin sulphate chains, the chain weights based on serine contents were calculated to be 16660 for the protein–polysaccharide and 18340 for the chondroitin sulphate–peptide. The latter value approaches the chain weight of the DNP-peptide deduced from its extinction.

Losses on hydrolysis in the presence of so much carbohydrate were assessed for serine and glycine, the principal amino acids of the faster electrophoretic fraction. Chondroitin 4-sulphate, from which amino acids had been largely removed by digestion with papain and subsequent treatment with dilute alkali (Muir, 1958), was hydrolysed alone and together with serine and glycine added in the approximate relative amounts found in the faster-moving fraction; 13% of added serine and 4·8% of added glycine were destroyed under the hydrolytic conditions used and their values in Table 1 have been corrected accordingly.

Hydrolysates of the electrophoretic fractions contained basic components that were eluted near the positions of lysine and histidine. None, however, coincided exactly with the positions of the authentic basic amino acids when these were chromatographed either alone or mixed with the hydrolysates. Other minor unidentified components that were observed did not interfere with the determination of known amino acids. Most of the unidentified components were reproduced on heating galactosamine in 6N-hydrochloric acid with equimolar amounts of glucuronolactone, acetic acid and sodium sulphate under conditions used to hydrolyse the protein–polysaccharides. They were not produced until the mixture was heated. Glucosamine when treated likewise also gave similar but not identical compounds that reacted with ninhydrin.

DISCUSSION

Protein–polysaccharides are generally obtained by disintegrating cartilage thoroughly by high-speed homogenization in water (Malawista & Schubert, 1958), followed by removal of rapidly sedimenting material to give a collagen-free product known as 'protein–polysaccharide light fraction' or PP-L fraction (Gerber *et al.* 1960). The method of extraction used here was notably different, because not only were neutral solutions of calcium chloride used instead of water but the cartilage was not disintegrated to anything like the same extent. After the sliced cartilage had been homogenized it still contained granules of intact cartilage and fibrous material. The extraction was thus very incomplete and may have selected more-soluble protein–polysaccharides, which are likely to be the smallest. This might explain why material prepared in this way had a different amino acid composition and contained less protein (Muir, 1958) than PP-L fraction from bovine nasal septum (Scheinthal & Schubert, 1963; Gregory *et al.* 1964; Anderson *et al.* 1965). These differences may, however, be partly due to a species difference, because PP-L fraction from pig metaphyseal cartilage had an amino acid composition (Castellani, Bonferoni, Ronchi, Ferri & Malcovati, 1962) less like that of PP-L fraction from bovine nasal

septum than of a protein–polysaccharide extracted from pig larynx and trachea by the present method (Muir, 1958).

PP-L preparations from bovine nasal septum may perhaps be mixtures of several compounds, because considerable differences in amino acid composition have been reported (Scheinthal & Schubert, 1963; Gregory *et al.* 1964; Anderson *et al.* 1965). The protein–polysaccharide from pig larynx was clearly heterogeneous also because it was divided on zone electrophoresis into two fractions with notably different analyses, although two distinct zones were not obtained. The faster fraction did, however, contain somewhat more sulphate and uronic acid than the slower fraction. Sulphate was determined by a method that had given good agreement (Muir, 1965) with the method of Picou & Waterlow (1963) using ^{133}Ba. The effective distance travelled by the polyanions was much greater than the apparent distance because of marked electroendosmosis towards the cathode in glass fibre at pH 7·2. This would explain why the rear of the band stayed near the origin. Since polyanions with a lower charge density than chondroitin sulphate moved towards the cathode in glass fibre at pH 7·2, any protein not covalently bound to polyanions would have moved far towards the cathode. Neither fraction contained any hydroxyproline (Woessner, 1961), and their amino acid compositions were unlike those of expected contaminants such as serum proteins, only traces of which were detected by immunological methods in the preparation before electrophoresis.

Both fractions were antigenic due to their protein moieties, because papain destroyed their antigenicity *in vitro* but hyaluronidase did not (Loewi & Muir, 1965). The fraction of higher electrophoretic mobility appeared to be a single antigen, which cross-reacted with protein–polysaccharides from other species, whereas the fraction of lower mobility contained a number of species-specific antigens (Loewi & Muir, 1965), suggesting that it contained several components.

The two fractions differed considerably in their protein content. The amino acid composition of the fraction of higher mobility was unusually simple, serine and glycine accounting for over half the amino acid residues (Table 1). Basic and sulphur-containing amino acids were apparently absent and also tyrosine, an amino acid known to enhance antigenicity (Sela, 1964).

Although somewhat polydisperse, the weight-average molecular weight (M_w) of this fraction was 230000 determined by sedimentation. Since it contained only about 2% of protein, the latter would at most be no larger than 4000–5000. It is therefore surprising, in view of its simple amino acid composition and small size, that this polypeptide was antigenic and should have evoked circulating antibodies for which this size range may be a lower limit (Campbell & Garvey, 1963), although perhaps not for the induction of delayed hypersensitivity (Leskowitz, Jones & Zak, 1966). It is possible that the antigenicity of the polypeptide is enhanced by being attached to chondroitin sulphate chains, which increase the size of the molecule. Chondroitin sulphate itself was not antigenic since chondroitin sulphate–peptide derived from the protein–polysaccharide did not inhibit antigen–antibody reactions (Loewi & Muir, 1965).

The M_w of the fraction of higher mobility was very much less than molecular weights of the order of 1×10^6–5×10^6 (Partridge, Davis & Adair, 1961) and 4×10^6 (Mathews & Lozaityte, 1958) reported for PP-L preparations. Its smaller size might explain why it was extracted without complete disintegration of the cartilage. It is possible to argue, however, that this fraction was merely a product of autolysis. On the other hand, the cartilage was completely fresh and was extracted with neutral hypertonic solutions of calcium chloride and not with water, which releases lysosomal proteases (Lucy, Dingle & Fell, 1961) that have an acid pH optimum (Fell & Dingle, 1963). Degradation products of lysosomal enzymes are therefore more likely in PP-L preparations. If degradation did occur, it was limited in extent because the product retained an antigenic determinant that cross-reacted with PP-L fractions from other species, and extensive proteolysis gave a much smaller product.

The fraction of higher mobility was distinct in almost lacking glucosamine. PP-L preparations contain significant amounts, usually attributed to the presence of some keratan sulphate, possibly as an integral part of the macromolecule (Partridge & Elsden, 1961; Gregory & Rodén, 1961). The hexose in the faster electrophoretic fraction, however, can be attributed to the terminal sequence of sugars that Rodén and his collaborators have shown attach chondroitin sulphate chains to serine residues in the protein moiety of PP-L fractions (Gregory *et al.* 1964; Lindahl & Rodén, 1966; Rodén, 1966; Rodén & Armand, 1966), there being two galactose residues at each linkage point.

If there were the same sequence of sugars at the linkage points in the fraction of higher mobility, then most of the serine residues would appear to be attached to chondroitin sulphate chains because there was at least twice as much hexose as serine in this fraction. The total serine content corrected for losses on hydrolysis should then provide an estimate of the average chain weight of the chondroitin sulphate chains in this protein–polysaccharide, which was thus calculated to be 16660.

That most of the serine residues were indeed attached to chondroitin sulphate chains is suggested by the somewhat similar values for the chain weight of the chondroitin sulphate–peptide calculated from its serine content as well as from the extinction of its DNP derivative. These values were 18340 and 19000–19500 respectively, both estimates giving number-average weights. The weight-average molecular weight of the chondroitin sulphate peptide was 19200 determined by sedimentation. Since this is close to the estimated number-average weights, the chondroitin sulphate chains may be reasonably uniform in length. These values are similar to those of Partridge *et al.* (1961) obtained by osmotic pressure measurements on bovine chondroitin sulphate–peptide and those of Kent & Stevenson (1965) calculated from the extinction of its DNP derivative.

Losses of serine under the conditions of hydrolysis were 13%. This is no greater than the losses of serine that occurred when ribonuclease was hydrolysed in the absence of carbohydrate (Hirs, Stein & Moore, 1954). Several minor basic components were observed after hydrolysis, but they could not be attributed to authentic basic amino acids. Although these unidentified basic compounds were reproduced when galactosamine and glucuronolactone were heated together under the conditions of hydrolysis, they accounted for very little of the galactosamine destroyed, most of which was converted into ammonia. Basic amino acids may not be altogether absent from the fraction of higher electrophoretic mobility, however, because, although its weight-average molecular weight was 230000 determined by sedimentation, dinitrophenylation showed that there was one available amino group in a weight of 47500.

In contrast, the fraction of lower electrophoretic mobility resembled PP-L preparations in some respects. It contained approx. 10% of protein and, compared with the whole protein–polysaccharide preparation obtained by the present method (Muir, 1958), its amino acid composition came to resemble that of PP-L fraction after the fraction of higher mobility had been removed. Aromatic, basic and sulphur-containing amino acids were present, and serine and glycine were not predominant as in the faster-moving fraction. This suggests that the latter is a minor component in cartilage, most of the less-soluble material resembling PP-L fraction being left in the cartilage by the present extraction procedure. A minor component of relatively low molecular weight such as this could be overlooked in a PP-L preparation.

The fraction of lower electrophoretic mobility contained a significant amount of glucosamine and correspondingly more hexose than the fraction of higher mobility. Its optical rotation was also somewhat lower. The glucosamine may be attributable to keratan sulphate, which contains equimolar amounts of galactose and glucosamine and has a very low optical rotation ($[\alpha]_D + 4°$; Meyer, Linker, Davidson & Weissman, 1953).

Despite these differences, however, the optical-rotatory-dispersion curves of both fractions were similar and consistent with those of flexible-chain polyelectrolytes (Jirgensons, 1966). The lack of helical structure might be expected from their rather high proline contents (Seifter & Gallop, 1966).

The high values for hexosamine recorded here may be due to the fact that, in the Elson–Morgan reaction, the distillation procedure of Cessi & Piliego (1960) gave values 11–13% above those obtained by a direct method (Kraan & Muir, 1957) with hydrolysates of chondroitin sulphate–peptide or the protein–polysaccharide of higher mobility. The Cessi & Piliego (1960) procedure has seldom been applied to chondroitin sulphate derivatives.

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