Biochem. J. (1967) **102**, 110

The Composition and Physicochemical Properties of Bovine Nasal-Septa Protein–Polysaccharide Complex

By MOLLIE LUSCOMBE AND C. F. PHELPS
Department of Biochemistry, University of Bristol

(*Received 2 May* 1966)

1. Protein–polysaccharide complexes were prepared in three different ways and the gross stoicheiometry of the complexes compared. 2. The neutral sugar content was ascertained and the possibility of a glycoprotein occurring with chondroitin sulphate and keratosulphate is discussed. 3. Physical data support a molecule of molecular weight $3{\cdot}2\times10^6$–$5{\cdot}8\times10^6$ with a roughly spherical domain and an average radius of gyration of 1390Å. Such a particle is highly solvated. The complex is heavily charged with the sulphate groups on the outside. 4. These findings are discussed in the light of the physiological role of protein–polysaccharide light fraction (PPL) in cartilage.

A complex of chondroitin sulphate and protein was separated from ox nasal-septa cartilage by Malawista & Shubert (1958). Later it was shown by Gerber, Franklin & Shubert (1960) that this complex could be resolved into two fractions by high-speed centrifugation, and these fractions were designated PPL* and PPH. Little analytical work has been performed on PPH and most interest has been centred on the composition and type of linkage of protein to polysaccharide in PPL.

The first part of the present paper enquires into the homogeneity of the complex, and then discusses the stoicheiometry of the components, with especial reference to the content of neutral sugars. The last part of the paper discusses some physical measurements.

The work throws some light on the properties of water-extracted PPL and suggests interpretations that may have relevance to understanding the role of mucopolysaccharides in the extracellular tissues.

MATERIALS AND METHODS

Extraction of protein–polysaccharide from cartilage

Cartilage from the nasal septa of 2-year-old cows was dissected free from perichondrial membranes and sliced finely. The extraction procedure followed closely that of Partridge, Davis & Adair (1961). The homogenate was spun for 30min. at $9500g_{av.}$ at 4° in an MSE 18 centrifuge and the supernatant was treated in one of three ways.

(1) The procedure of Partridge *et al.* (1961) was used followed by high-speed centrifugation in an MSE 50 centrifuge at $96000g_{av.}$ according to the method of Gerber *et al.* (1960) to give PPL and PPH fractions.

(2) The supernatant was ultrafiltered on grade-5 sintered-glass filters (maximum pore sizes 1·35–1·7μ). Toluene was added as a bacteriostatic agent. Ultrafiltration proceeded at 4° with frequent stirring to prevent blocking of the pores. The residue was washed with twice its volume of water and then dialysed at 4° for 2 days against frequent changes of distilled water. After dilution with sufficient KCl solution to give a concentration of 0·5% of protein–polysaccharide in 0·15M-KCl it was centrifuged at high speed as described for the preparation of PPL. The resulting supernatant, after dialysis to remove KCl, was freeze-dried and used as the ultrafilter-residue fraction.

(3) A 35ml. volume of 1% (w/v) cetylpyridinium chloride solution was added to 300ml. of the supernatant plus 300ml. of water and left for 16hr. The precipitate was recovered by centrifugation and washed twice with 400ml. of 95% (v/v) ethanol, saturated with potassium acetate, to remove precipitant. The washed precipitate was stirred with 200ml. of water for 2hr. at 4° and then centrifuged. To the supernatant were added twice its volume of ethanol and 5g. of potassium acetate. The precipitate of protein–polysaccharide was collected by centrifugation, dialysed and freeze-dried. This fraction was the cetylpyridinium-precipitated complex.

Dry weights

Results are expressed as percentage of the dry weight obtained by drying solutions of the complex at 105° for 20hr. The moisture content of freeze-dried material was variable, so that dry material was not a suitable starting material for analysis; instead, portions of a solution whose dried weight had been determined at 105° were weighed out and accurately diluted for analysis.

Chemical measurements

Hexosamines. The complex was hydrolysed in 4N-HCl (1ml. of acid/mg. of complex) either under reflux for 6hr. or in sealed tubes immersed in boiling water for 8hr. The hydrolysates were evaporated at 40° in a rotary evaporator or in a vacuum desiccator over NaOH pellets. Residues

* Abbreviations: PPL, protein–polysaccharide light fraction; PPH, protein–polysaccharide heavy fraction.

were neutralized before estimation either by the method of Neuhaus & Letzman (1957) or by that of Rondle & Morgan (1955), except that the incubation with acetylacetone was carried out at 90° for 45min. in the latter method. Similar results were obtained from both procedures. Standards of both glucosamine and galactosamine were used. The two hexosamines were separated by the method of Gardell (1953).

Hexuronic acid. This was estimated by the method of Bitter & Muir (1962) or the decarboxylation method of Ogston & Stanier (1951).

Results from the micro-manometric method and the carbazole method differed by less than 3%. Internal standards in the carbazole method gave 99·5% recovery, so that the protein present did not affect the reaction. Neutral sugars will contribute to the reading but this was estimated to be only about 0·5% or less.

Sulphate. Estimations of sulphate on samples, which had been digested with HNO_3 and H_2O_2 (Foster, Harrison, Inch, Stacey & Webber, 1963) or with HNO_3 at 140° (Paulson, 1953), were by the method of Jones & Letham (1956).

Protein. This was estimated by the biuret method as described by Gornall, Bardawill & David (1949), with bovine serum albumin as standard, or by Millar's (1959) modification of the Folin–phenol method, with crystalline lysozyme as standard.

The Folin–phenol method consistently gave lower results than the biuret method. Although colour development in the biuret reaction is not identical with all proteins the deviations are less than with the Folin–phenol method, in which part of the colour is derived from the reaction of tyrosine and tryptophan with the phosphomolybdic acid–phosphotungstic acid reagent.

The tyrosine/tryptophan ratio of a PPL preparation, calculated from the ultraviolet spectrum of a 0·22% solution in 0·1N-NaOH, was 2·02 (Beavan & Holliday, 1952). The amounts of tyrosine and tryptophan in the complex were 0·78% and 0·43% respectively, whereas lysozyme contains eight times as much. The biuret values are therefore a more reliable estimate of the protein content of the complex.

Nitrogen. This was determined by the micro-Kjeldahl procedure with selenium catalyst.

Sialic acid. This was estimated by the thiobarbituric method of Warren (1959).

Hydroxyproline. This was estimated by the method of Miyada & Tappel (1956).

Reagents used. These were of micro-analytical reagent grade or AnalaR wherever possible. Water was double glass-distilled.

Separation and estimation of neutral sugars

Paper chromatography. Samples (30–50mg.) of protein–polysaccharide were hydrolysed in 0·5N-H_2SO_4 for various times. The hydrolysates were neutralized with $Ba(OH)_2$ and the precipitate of $BaSO_4$ was removed by centrifugation. Hexosamines and amino acids were removed from the supernatant by passing it through a Dowex 50 (H^+ form; 200–400 mesh) column (4cm.×0·7cm.). The column was washed with 2ml. of water and the supernatant and washings were evaporated to dryness in a vacuum desiccator. The resin did not affect the sugar pattern and a 96% recovery of a known mixture of sugars, at concentrations similar to those found in hydrolysates, was obtained when the mixture dissolved in hot acid was treated in the same way as the hydrolysate.

The residue after evaporation was dissolved in 0·05ml. of water and 0·01ml. portions were spotted on Whatman no. 1 filter paper that had been sprayed with 0·1M-phosphate buffer, pH5, and dried. The chromatograms were developed by descending chromatography for 24hr. with as solvent butan-1-ol–acetone–water (4:5:1, by vol.) (Meier & Wilkie, 1959). After drying, the papers were sprayed with 3% *p*-anisidine hydrochloride in butan-1-ol–ethanol–water (4:1:1, by vol.) containing a trace of $SnCl_2$, dried and heated at 100° for 5min. To estimate the amount of sugars present in the sample, various known quantities of standards were run concurrently.

The identity of the sugars in the hydrolysates was confirmed by running in two more solvents: an acidic solvent, butan-1-ol–acetic acid–water (20:5:11, by vol.) (Jermyn & Isherwood, 1949), and a basic solvent, pyridine–ethyl acetate–water (11:40:6, by vol.) (Block, Durrum & Zweig, 1958).

Column chromatography. Cellulose phosphate powder was suspended in acetone and packed under pressure to give a column 50cm.×1·25cm. The column was equilibrated with the eluting solvent, butan-1-ol–acetone–water (4:5:1, by vol.). A residue that had been obtained by treating a 6hr. hydrolysate of 220mg. of PPL as described in the preceding section was dissolved in 0·5ml. of water, and 0·3ml. was put on the column and eluted with a total of 880ml. of solvent; 3ml. fractions were collected.

The location of the sugars in the fractions was made by spotting a small quantity from each tube on phosphate-sprayed paper and developing the chromatogram as described in the preceding section. A 90% recovery of sugars was obtained.

With fucose–xylose and mannose–glucose there was slight overlapping, and the tubes where overlapping took place were pooled separately and estimated for both the sugars present. The values obtained were added to those found for the main fraction of each sugar.

Galactose and mannose were estimated by the anthrone method of Yemm & Willis (1954) by using a 6·5min. heating period, fucose and xylose by the cysteine–H_2SO_4 reaction of Dische as described by Ashwell (1957), and glucose with glucose oxidase (Huggett & Nixon, 1957).

The presence of other sugars interfered with the estimation of galactose and fucose in the complex. Colours developed by 100μg. of these other sugars were found to be equivalent to the following μg. of galactose: galactosamine, 2·5; glucose, 138; mannose, 100; glucosamine, 1·2; glucuronic acid, 4·2; xylose, 8; and the following μg. of fucose: galactosamine, 0·78; glucosamine, 0·37; glucuronic acid, 1·05; galactose, 1·1. Interference from xylose was eliminated by allowing the tubes to stand for 16hr. after the addition of cysteine. The colours were additive and corrections were made for the values of galactose and fucose obtained.

Physical measurements

Electrophoresis. Electrophoresis was carried out in a horizontal-electrophoresis tank with Oxoid cellulose acetate strips. A constant voltage of 400v for 75min. at 4° in

buffers of constant ionic strength (I 0·1) was used. The presence of mucopolysaccharide material was shown by staining with Alcian Blue. The effect of electroendosmosis was measured by running samples of dextran under identical conditions.

Viscosity. Viscosity determinations were made in a Couette viscometer built to the specification of Ogston & Stanier (1953). Velocity gradients were between 0·09 and 5·4sec.$^{-1}$ and the temperature 25° or 20°.

Ultracentrifugation. A Spinco model E analytical ultracentrifuge with a schlieren optical system was used. The constant-temperature control was set at 20° and the maximum speed of 59780 rev./min. was attained.

PPL was dissolved in a saline–phosphate buffer composed of 0·2M-NaCl in 0·01M-phosphate buffer, pH7. The sedimentation coefficient at zero concentration was obtained by the extrapolation of $1/S_{20,w}$ against concentration. The experimental values were corrected for $S_{20,w}$ according to the equation of Svedberg & Pederson (1940, p. 35).

Partial specific volume. The partial specific volume was calculated from density measurements made at 20° with a 10ml. density bottle.

Light-scattering. This was measured in a Sofica (Le Mesnil Saint Denis, Seine-et-Oise, France) Photo Gonio diffusometer at a series of angles between 30° and 150° with unpolarized light of wavelength 546mμ. Solutions of PPL used covered the range of concentrations from $11{\cdot}36\times10^{-5}$ to $0{\cdot}788\times10^{-5}$g./ml. and were clarified by centrifuging at 17000g (Servall Superspeed centrifuge, SS4 rotor). The solvent was the same saline–phosphate buffer as used for ultracentrifugation. The results were plotted by the method of Zimm (1948).

Specific refractive increment. This was determined on a differential refractometer (Polymer Consultants Ltd., Colchester, Essex). The apparatus was in a room at 25°. It was calibrated for mercury light of wavelength 546mμ with sucrose solutions by using the values reported by Gosting & Morris (1949).

RESULTS

Homogeneity of complex

The physical methods currently available for testing homogeneity were incapable of giving unambiguous information. Pilot experiments, involving (*a*) centrifugation on sucrose and caesium chloride density gradients and (*b*) constant solvent solubility tests with ethanol and potassium acetate, both revealed an essentially homogeneous, although polydisperse, component (Luscombe, 1965). Thus another approach was made. This assumed that material prepared by three different methods should give identical values if the complex is an integral unit, whereas discrepancies would be found in the proportions of the main components if the complex were inhomogeneous. Analyses of the complex prepared by three methods, namely precipitation as the potassium salt by ethanol (PPL), ultrafiltration (ultrafilter residue) and cetylpyridinium chloride precipitation (cetylpyridinium-precipitated complex), are given in Table 1.

All three samples were made from an original extract prepared by homogenization for 4min. in a Waring Blendor at 14000 rev./min. followed by centrifugation at 9500g for 20min. The only other method available for the extraction of the complex involved the use of a percussion mortar at −15° and dealt with very small quantities of material.

The hexuronic acid/hexosamine ratios are similar in all three preparations. Protein is the most variable constituent, especially between PPL and ultrafilter residue. Toluene was added to the homogenate during ultrafiltration, so that it is unlikely that degradation by bacteria occurred. Protein degradation caused by the presence of endogenous enzymes cannot be completely ruled out, but little would be expected in such a relatively acellular tissue, and to minimize their effect the homogenate was kept at 4°.

Composition of the complex

Analysis of the whole complex. (*a*) Hexosamines. The optimum time for hydrolysis of the complex with 4N-hydrochloric acid under reflux was 6–9hr. (Fig. 1). Increased time of hydrolysis caused

Table 1. *Comparison of the compositions of cartilage protein–polysaccharide prepared by three methods*

The preparation of the protein polysaccharides is given in the Materials and Methods section. UFR, ultrafilter residue; CPP, cetylpyridinium-precipitated complex.

	Composition (% of dry wt.)		
	PPL	UFR	CPP
Hexuronic acid	25·2	24·6	24·0
Hexosamine	26·7	26·1	24·5
Hexosamine/hexuronic acid molar ratio	1·14	1·16	1·10
Protein (biuret)	20·3	16·8	17·9
Galactose (anthrone)	4·6	4·3	5·0
Sulphur	4·5	4·4	—
Sialic acid	1·11	0·95	0·97
Nitrogen	5·2	4·4	—

decomposition of the hexosamines; glucosamine is slightly more stable, as shown by the increase in the glucosamine/galactosamine ratio after 9hr. hydrolysis. Heating in sealed tubes at 100° for 8hr. also gave satisfactory hydrolysis. An internal standard of galactosamine gave a 98% recovery by this method.

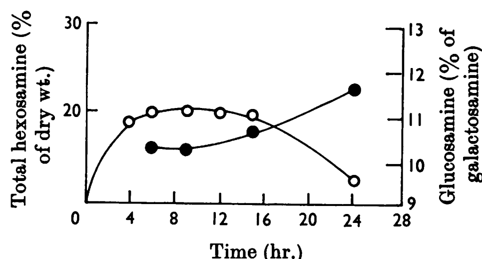

Fig. 1. Time-course of the release of hexosamines from PPL during hydrolysis with 4N-HCl. ○, Total hexosamine set free, measured as glucosamine, recorded as a percentage of the dry weight of the sample; ●, amount of glucosamine recorded as a percentage of the amount of galactosamine.

(*b*) Hydroxyproline. The hydroxyproline content of PPL and ultrafilter-residue preparations was 0·1% or less, whereas PPH gave values of 3·0–5·0%, no allowance being made for interference from other amino acids.

A summary of the analyses of the complex is given in Table 2. These values were used to calculate the percentage composition of PPL. PPH dissolves in water with difficulty and the resulting solution does not appear to be homogeneous. This would account for the wider range found in these results than with PPL.

Analysis for neutral sugars. The time-course for the release of galactose, glucose, mannose, xylose and fucose from PPL is shown in Fig. 2. Galactose is the only sugar that appears in increasing amounts during hydrolysis beyond 6hr. Possibly keratosulphate is hydrolysed at a different rate from the glycoprotein and glycopeptide linkages.

Table 2. *Analyses of the PPL and PPH fractions of bovine nasal cartilage*

Experimental details are given in the text. All results are the average of at least two analyses. Where more than one preparation was analysed the range of values obtained is given in parentheses.

		Composition of PPH (% of dry wt.)	Composition of PPL (% of complex dry wt.)	Composition of PPL (μmoles/g.)
Hexuronic acid				
(as free acid)		9·7 (9·3–10·7)	25·0 (24·0–25·6)	1300
(*A*) (as potassium salt)			30·0	
Total hexosamine				
(as free base)		10·7 (9·5–11·9)	26·3 (25·6–26·7)	1480
(*B*) (as *N*-acetylhexosamine)			32·6	
Galactosamine			23·7	1320
Glucosamine			2·9	160
Protein				
(*C*) Biuret		61·6 (61·2–62·0)	19·8 (18·8–20·3)	
Folin–phenol		42·0 (41·4–42·7)	14·7 (13·6–15·2)	
Amino acids				1590
Neutral sugars				
(*D*) Galactose	on whole complex	7·2	4·4 (4·2–4·6)	245
(*D*) Fucose	on whole complex	0·09	0·25 (0·23–0·29)	15
(*D*) Glucose	chromatographically separated from 6hr. hydrolysates	1·10	0·17 (0·15–0·19)	9
(*D*) Mannose	chromatographically separated from 6hr. hydrolysates	0·28	0·36 (0·35–0·38)	20
(*D*) Xylose	chromatographically separated from 6hr. hydrolysates	0·09	0·41 (0·38–0·44)	27
Fucose	chromatographically separated from 6hr. hydrolysates	0·04	0·10 (0·09–0·11)	
Galactose	chromatographically separated from 6hr. hydrolysates	1·5	2·00 (1·95–2·08)	
Sialic acid				
(*E*) (as *N*-acetyl derivative)		0·74	1·14 (1·11–1·19)	37
Sulphur				
(as S)		2·15 (1·7–2·6)	4·52 (4·30–4·70)	1420
(*F*) (as KSO_3)			16·6	
Nitrogen			5·1 (4·9–5·2)	
Ash			22·7 (22·4–22·8)	
Total $A+B+C+D+E+F$			105·7	
Less water of condensation between sugar residues			5·5	
			100·2	

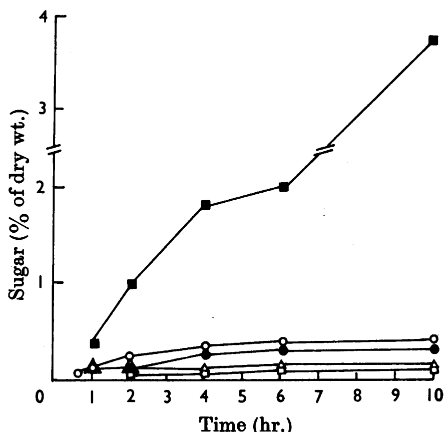

Fig. 2. Release of sugars during acid hydrolysis of PPL. Experimental details are given in the text. ■, Galactose; △, glucose; ○, xylose; ●, mannose; □, fucose.

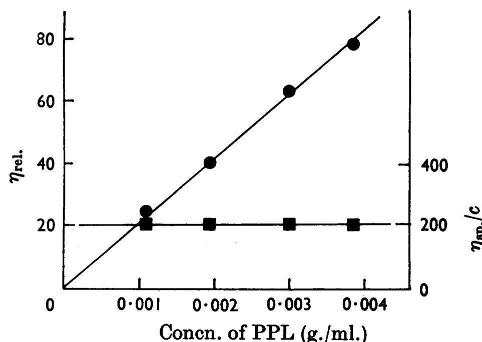

Fig. 3. Viscosity of solutions of PPL in water. Measurements were made at 25°. ●, Relative viscosity; ■, reduced viscosity.

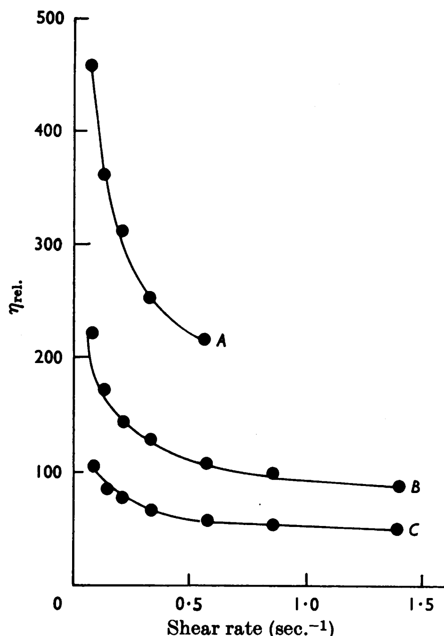

Fig. 4. Change of relative viscosity with increasing shear rate of solutions of ultrafilter residue in water at the following concentrations: *A*, 0·194%; *B*, 0·129%; *C*, 0·097%. Measurements were made at 25°.

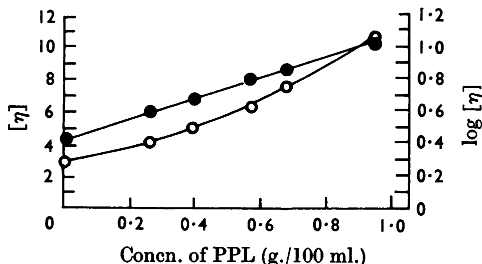

Fig. 5. Estimation of the intrinsic viscosity of PPL in saline–phosphate buffer (0·2M-NaCl in 0·01M-phosphate buffer, pH 7). Measurements were made at 20°. ○, Reduced viscosity; ●, log (reduced viscosity).

A comparison of values obtained by hydrolysis of PPL and PPH is given in Table 2. The most notable difference between the two is the high concentration of glucose in PPH while the xylose concentration is only one-fifth of that in PPL. Much of the PPH glucose was easily hydrolysed, approx. 90% of that present being released in 2hr.

Physical data

Viscosity. The viscosity of the complex was determined in a Couette viscometer by measuring the angular deflexion of the torsion head required to return the torsion wire to zero when a shear was applied to the solution. A plot of these angles for shear rates of 0·92–5·4sec.$^{-1}$ for solutions of PPL in water gave straight lines. There was no sign of anomalous viscosity. The change in relative viscosity with concentration and the reduced viscosity are shown in Fig. 3. The reduced viscosity is independent of the concentration over the range used.

A supernatant that had been ultrafiltered and then spun at 96000$g_{av.}$ for 1hr. without added salt showed definite anomalous viscosity. The relative viscosities and varying shear rates for three concentrations are shown in Fig. 4.

Although the reduced viscosity was independent of the concentration of PPL when water was the solvent, a marked dependence was found with the

saline–phosphate buffer used for ultracentrifugation (Fig. 5). The intrinsic viscosity was 2·70 dl./g. in 0·2 M-sodium chloride in 0·01 M-phosphate buffer. The plot of change in viscosity with concentration is not linear at low concentrations. The addition of salts to give quite low ionic strengths results in a marked lowering of viscosity. Fig. 6 illustrates the effect of increasing the ionic strength of a 0·5% solution of PPL with phosphate buffer, pH 7·4.

The effect of pH on viscosity at I 0·2 is shown in Fig. 7. The reduced viscosity decreases as the pH is lowered. The composition of the buffer also plays a part in determining the viscosity; thus in glycine and tris buffers the relative viscosities are higher than in other buffers of similar pH, probably owing to association of the complex.

Electrophoresis. The pH of minimum mobility of both PPL and deproteinized complex was 1·1–1·2. The removal of sulphate groups with limpet sulphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.) slightly raised the apparent isoelectric point.

Ultracentrifugation. The concentration-dependence of the sedimentation coefficient is shown in Fig. 8. The sedimentation coefficient at zero concentration determined by extrapolation of $1/S$ against c to $c=0$ was 18·18 s.

Partial specific volume. The determination of $\bar{v}$ experimentally gave values of 0·675–0·685. This was in agreement with the calculated value of 0·68 based on the complex containing 20% of protein.

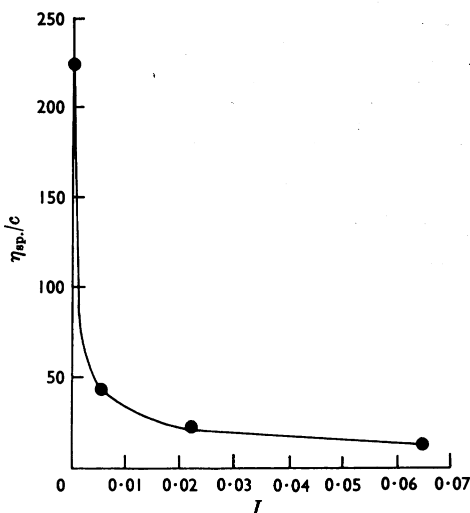

Fig. 6. Effect of ionic strength on the reduced viscosity of 0·5% solutions of PPL in sodium phosphate buffer, pH 7·4 (of different ionic strengths). Measurements were made at 25°.

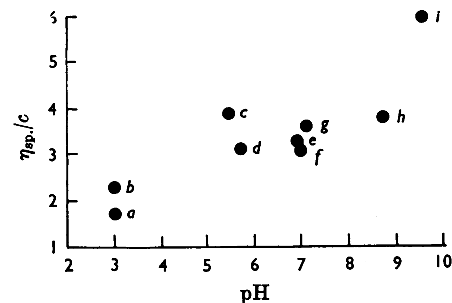

Fig. 7. Dependence of the reduced viscosity of PPL on the pH of the solution. Buffers were all at I 0·2. The buffer constituents were: *a*, formic acid–NaOH; *b*, HCl–glycine; *c*, acetic acid–NaOH; *d* and *e*, cacodylic acid–NaOH; *f*, NaH_2PO_4–Na_2HPO_4; *g*, tris–HCl; *h*, aq. NH_3–HCl; *i*, NaOH–glycine. Measurements were made at 25°.

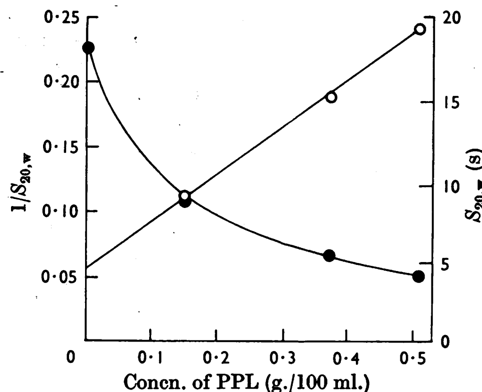

Fig. 8. Concentration-dependence of the sedimentation coefficient of PPL in saline–phosphate buffer (0·2 M-NaCl in 0·01 M-phosphate buffer, pH 7). ●, Variation of $S_{20,w}$ with concentration; ○, variation of $1/S_{20,w}$ with concentration.

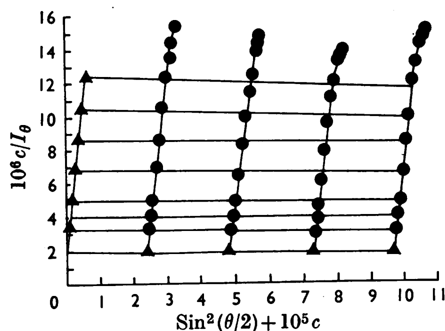

Fig. 9. Light-scattering Zimm plot for PPL in saline–phosphate buffer (0·2 M-NaCl in 0·01 M-phosphate buffer, pH 7). ●, Experimental points; ▲, points obtained by extrapolation.

Light-scattering. The specific refractive increment dn/dc was 0·170 g./ml. This leads to a value of K, equal to $2\pi^2 n_0^2 (dn/dc)^2 / N\lambda^4$, of $1{\cdot}93 \times 10^{-7}$. Results obtained for one sample of PPL are shown in Fig. 9, expressed in the form of a Zimm plot.

DISCUSSION

Homogeneity

The preparation of the complex entails two operations: (*a*) conversion of the comminuted cartilage into a form from which the soluble chondromucoprotein can be extracted and (*b*) the subsequent purification of the solubilized material. The extraction method of Partridge *et al.* (1961) used ethanol at room temperature. It was thought that this reagent might either cause denaturation of the protein part of the complex or cause externally denatured protein molecules to be firmly adsorbed on the complex. Scott's (1960) method of selectively precipitating mucopolysaccharides from solution with long-chain quaternary nitrogen anions seemed an attractive possibility. The high concentration of protein present in the material led to difficulties in the resolubilization of the polysaccharide complex that could only be overcome by the use of ethanol. A third method of preparing the complex consisted in adapting the sintered-glass filters of Ogston & Stanier (1951) and preparing an ultrafilter residue. As shown in Table 1 the three complexes are very similar in chemical composition. In addition, neither careful fractionation with ethanol nor density-gradient centrifugation revealed any gross inhomogeneity, and it was concluded that the preparation of Malawista & Shubert (1958) as modified by Gerber *et al.* (1960) produced material of reproducible constitution that bore a close relationship to the material as it occurred physiologically in cartilage.

Kaplan & Meyer (1959) showed that the ratio of chondroitin sulphate A to keratosulphate and chondroitin sulphate C can vary in cartilage of different age, but material from only one age group of animal has been used in these investigations.

Stoicheiometry

The accurate measurement of the concentration of the individual components of the complex presents many problems, the chief of which resides in the unspecificity of the analytical reagents. Care has been taken in the present work to cross-check analytical practices either by using two unrelated methods or by the incorporation of internal standards.

Within these limitations the final analysis accounting for 100·2% of the weight of the complex is given in Table 2. The amino acids are calculated on a protein content of 19·8% by using the percentage composition published by Campo & Dziewiatkowski (1962) for PPL.

Size and shape of the molecule

Estimations based on sedimentation and viscosity data. The values of $S^0_{20,w}$ and $[\eta]$ have been used to calculate the molecular weight of the complex. The viscosity of the solvent, η_0, was 0·01024 poise and the density, ρ, was 1·0077.

The hydrodynamic model on which to base the calculation of M and V' is limited by the available data and the suitability of the mathematical models proposed. The β function of Sheraga & Mandelkern (1953), which is based on impenetrable equivalent hydrodynamic ellipsoids, leads to lower estimates of particle volume if the molecule is penetrated by solvent (Fessler & Ogston, 1951). When applied to non-ionic polymers, treated as an equivalent hydrodynamic sphere, the β function is estimated as $2{\cdot}12 \times 10^6$, which is not in accordance with experimental data on polymers of known molecular weight, which give values of $2{\cdot}3 \times 10^6$–$2{\cdot}6 \times 10^6$. The hydrodynamic theory of Kirkwood & Riseman (1948) and Mandelkern & Florey (1952) requires a value of $2{\cdot}5 \times 10^6$. However, the complex under examination is a polyelectrolyte and the viscosity would be expected to exhibit some electroviscous effects not allowed for in the other hydrodynamic theories. The high-molarity salt solutions in which measurements were made should reduce these effects to a minimum.

In the absence of a more suitable model the values have been calculated for a series of β values and other functions of the ellipicity J (the axial ratio a/b, namely axis of revolution/equatorial axis). Values for the various functions of J have been taken from the Table given by Ogston (1953). Table 3 gives the calculated values.

Table 3 (column 3) shows that the molecular weight does not vary greatly in the range J equal to 1–5. However, the calculated value of $d(1/S)/dc$ of $0{\cdot}323 \times 10^{13}$ for J equal to 1 is most nearly in agreement with the experimental value of 0·366. A source of error in this calculation is the value taken for K. The median value is 1·8, but values up to 3 have been recorded. Thus by increasing the value for K it is possible to raise the values obtained corresponding to J equal to 2, 3 and 5. With K equal to 3, the value for J equal to 5 becomes 0·349. For all values of J above 5 the calculated $d(1/S)/dc$ falls far short of the experimental value even with the upper limit value for K. Values of J in the range 1–5 best fit the available data, indicating a spherical or slightly ellipsoidal overall shape for the particle. These values also lie within the region expected for random coils.

Table 3. *Summary of results obtained from hydrodynamic data*

Column (2): values of β function corresponding to J; column (3): calculated from the formula of Sheraga & Mandelkern (1953); column (4): calculated from $S^0_{20,w}$; and $[\eta]$ according to the method of Ogston (1953) ($\zeta=0.667$ and $K=1.8$); column (5): calculated by equating the molecular volume with the volume of an ellipsoid; column (6): calculated values for M were inserted into the Svedberg equation to estimate D (Svedberg & Pederson, 1940, p. 5); column (7): estimated from the value of D (Ogston, 1953).

(1) J	(2) $10^{-6}\beta$	(3) $10^{-6}M$	(4) $10^{-13}\times\frac{d(1/S)}{dc}$	(5) a (Å)	(6) $10^7 D$ (cm.²/sec.)	(7) V' (ml./g.)
1	2·12	3·57	0·323	98·7	0·393	106
2	2·13	3·55	0·303	156·4	0·396	91·6
3	2·16	3·48	0·274	203·5	0·404	72·9
5	2·23	3·32	0·209	281·7	0·423	46·2
10	2·41	2·95	0·109	432·9	0·476	19·7
20	2·64	2·57	0·048	651·8	0·547	6·86
50	2·97	2·16	0·013	1133	0·652	1·50
100	3·22	1·90	0·005	1727	0·736	0·45

For values of J in the range 1–5 the estimated hydrodynamic volume, V', lies in the range 106–46ml./g. The particle is therefore highly solvated even in 0·2M-sodium chloride. In water the highly charged chains will be extended to give a less dense and more rigid particle with a very highly solvated domain, and thus very high viscosity (Fig. 6).

Estimations based on light-scattering data. The mean molecular weight obtained from three series of experiments was $5\cdot8\times10^6$. The dissymmetry factor, I_{45}/I_{135}, was 2·8 and was not concentration-dependent. The second virial coefficient, B, obtained by extrapolating to $\theta=0°$ is negligible, indicating that there can be little or no interaction between particles at the concentrations used. The limiting slope of the plot of $c=0$ has been used to calculate the Z-average radius of gyration of 1390Å. Plots of the values of the scattering factor $P(\theta)$, also obtained from this slope, against $\sin(\theta/2)$ indicated that the shapes of the particles more nearly approximate to those of random coils of spherical overall shape.

Light-scattering, unlike sedimentation and viscosity, will give results corresponding to the undistorted hydrated molecule, but is prone to errors arising from polydispersity since the larger particles give increased light-scattering at low angles. Thus a higher estimate for M would be expected from light-scattering data as PPL was shown to be polydisperse by gradient centrifugation.

The values for M found fall within the region of those of Mathews & Lozaityte (1958), but are higher than those of Bernardi (1957). The latter author, however, used material obtained in much smaller yield than ours. The data of Mathews & Lozaityte (1958) suggested long rods whereas that of Bernardi (1957) suggested coils. Our light-scattering and viscosity data do not support the presence of long rods. The lack of non-Newtonian viscosity also rules out long coils, since the particle present in PPL behaves as an aggregate of random coils.

However, non-Newtonian viscosity was found with ultrafilter residue that had not been centrifuged in saline to remove the heavy fraction (Fig. 4). Such material is thought to contain much larger aggregates, which interact at the concentrations used, probably stabilized by the extra protein present. This material may be analogous to that with molecular weight 50×10^6 reported by Mathews & Lozaityte (1958).

Structural and physiological implications

Cartilage consists of collagen in intimate association with polysaccharide material, which can be extracted and divided into two fractions, namely PPL and PPH (Gerber *et al.* 1960). Very little is known about the composition and physical make-up of the latter. The high protein content is made up of about 60% of collagenous protein and it also contains easily hydrolysable glucose. It was shown by Rotstein, Gordon & Shubert (1962) that the insoluble residue left after the aqueous extraction of protein–polysaccharide complex contained, besides collagen, material containing hexosamine and a high percentage of neutral sugars. PPH probably represents part of this ground substance, with some PPL that had been trapped during ultracentrifugation.

PPL was shown to contain, in addition to chondroitin sulphate linked to protein, some keratosulphate (Gregory & Rodén, 1961). Evidence for its occurrence in the PPL used in the present work was found during work on enzymic degradation. Examination of the μmoles of the constituents/g.

of PPL (Table 2) shows a large excess of galactose over glucosamine. Gregory, Laurent & Rodén (1964) isolated glycopeptides by the enzymic degradation of PPL that contained, besides galactosamine and glucuronic acid, galactose and serine. Evidence was also presented for the presence of xylose, the linkage of polysaccharide to protein being through a galactose–galactose–xylose–serine linkage. The chain weight of chondroitin sulphate has been given as $2\cdot2\times10^4$–$2\cdot8\times10^4$ (Mathews, 1956; Partridge *et al.* 1961; Buddecke, Kröz & Lanka, 1963). Taking $2\cdot5\times10^4$ as an average chain weight, the value of 46 disaccharide units/chain is obtained. Thus there should be 1 mole of linkage residues for every 46 moles of glucuronic acid. Since 1 g. of PPL contains 1300 μmoles of glucuronic acid there should be 28 μmoles of linkage compound, i.e. 28 μmoles of xylose and 56 μmoles of galactose. We found 27 μmoles of xylose and 245 μmoles of galactose.

Taking into account the complement of sugars required to link chondroitin sulphate to protein, there remains an excess of uncommitted sugars that points to the presence of glycoprotein. Mannose, fucose and sialic acid, the last two of which are found exclusively in terminal positions of glycoproteins, occur in high enough concentration to render this suggestion tenable.

Obviously the molecular weight of the complex will reflect the contributions of these components. Previous reports of M of the crude extracted material range from 1×10^6 (Webber & Bayley, 1956) to 4×10^6–50×10^6 (Mathews & Lozaityte, 1958). The value of $3\cdot2\times10^6$–$5\cdot2\times10^6$ reported in the present paper for PPL of nasal septa is the first attempt to characterize the size of the complex as prepared by Gerber *et al.* (1960). This relates to a macromolecule probably composed of aggregates of protein cores with randomly coiled chondroitin sulphate chains, offering a tangled three-dimensional network in which sufficient space may be found to trap sterically both keratosulphate and glycoprotein. Since keratosulphate cannot be freed except by enzymic degradation the degree of entanglement must be considerable. The fact that protein can be removed by precipitation with phosphotungstic acid (Webber & Bayley, 1956) or cetylpyridinium chloride (Buddecke *et al.* 1963), resulting in material with much lower molecular weights, 1×10^6 and $5\cdot5\times10^5$ respectively, supports the idea that PPL exists as an aggregate. However, the preparation obtained by Gerber *et al.* (1960) by using a mild extraction procedure must produce material akin to that found physiologically, indicating a close association of protein–polysaccharide material with a glycoprotein.

The data on the effect of pH on viscosity and the electrophoretic measurements on various chondroitin sulphate preparations both with and without protein and with diminished sulphate content suggest strongly that the sulphate groups are on the outside of the aggregates. It is evident that such a complex will be highly charged at physiological pH, and such a charge may well stabilize the configuration of the aggregate. Such highly charged particles present in extracellular tissue must also affect ion distribution.

M. L. thanks the Medical Research Council for an assistantship and the Arthritis and Rheumatism Council for the loan of apparatus.

REFERENCES

Ashwell, G. (1957). In *Methods in Enzymology*, vol. 3, p. 103 and p. 90. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Beavan, G. H. & Holliday, E. R. (1952). *Advanc. Protein Chem.* **7**, 375.
Bernardi, G. (1957). *Biochim. biophys. Acta*, **26**, 47.
Bitter, T. & Muir, H. M. (1962). *Analyt. Biochem.* **4**, 330.
Block, R. J., Durrum, E. L. & Zweig, G. (1958). *The Manual of Paper Chromatography and Paper Electrophoresis*, p. 172. New York: Academic Press Inc.
Buddecke, E., Kröz, W. & Lanka, E. (1963). *Hoppe-Seyl. Z.* **331**, 196.
Campo, R. D. & Dziewiatkowski, D. D. (1962). *J. biol. Chem.* **237**, 2729.
Fessler, J. H. & Ogston, A. G. (1951). *Trans. Faraday Soc.* **47**, 667.
Foster, A. B., Harrison, R., Inch, T. D., Stacey, M., & Webber, J. M. (1963). *J. Amer. chem. Soc.* **85**, 2279.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Gerber, B. R., Franklin, E. C. & Shubert, M. (1960). *J. biol. Chem.* **235**, 2870.
Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Gosting, L. J. & Morris, M. S. (1949). *J. Amer. chem. Soc.* **71**, 1998.
Gregory, J. D., Laurent, T. C. & Rodén, L. (1964). *J. biol. Chem.* **239**, 3312.
Gregory, J. D. & Rodén, L. (1961). *Biochem. biophys. Res. Commun.* **5**, 430.
Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12P.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Jones, A. S. & Letham, D. S. (1956). *Analyst*, **81**, 15.
Kaplan, D. & Meyer, K. (1959). *Nature, Lond.*, **183**, 1267.
Kirkwood, J. G. & Riseman, J. (1948). *J. chem. Phys.* **16**, 565.
Luscombe, M. (1965). Ph.D. Thesis: University of Bristol.
Malawista, I. & Shubert, M. (1958). *J. biol. Chem.* **230**, 535.
Mandelkern, L. & Florey, P. J. (1952). *J. chem. Phys.* **20**, 212.
Mathews, M. B. (1956). *Arch. Biochem. Biophys.* **61**, 367.
Mathews, M. B. & Lozaityte, I. (1958). *Arch. Biochem. Biophys.* **74**, 158.
Meier, H. & Wilkie, K. C. B. (1959). *Holzforschung*, **13**, N.T.6.
Millar, G. L. (1959). *Analyt. Chem* **31**, 964.

Miyada, D. S. & Tappel, A. L. (1956). *Analyt. Chem.* **28**, 909.

Neuhaus, O. W. & Letzman, M. (1957). *Analyt. Chem.* **29**, 1230.

Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.

Ogston, A. G. & Stanier, J. E. (1951). *Biochem. J.* **49**, 591.

Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.

Partridge, S. M., Davis, H. F. & Adair, G. S. (1961). *Biochem. J.* **79**, 15.

Paulson, S. (1953). *Acta chem. scand.* **7**, 325.

Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.

Rotstein, J., Gordon, M. & Shubert, M. (1962). *Biochem. J.* **85**, 614.

Scott, J. E. (1960). *Meth. biochem. Anal.* **8**, 145.

Sheraga, H. A. & Mandelkern, L. (1953). *J. Amer. chem. Soc.* **75**, 179.

Svedberg, T. & Pederson, K. O. (1940). *The Ultracentrifuge.* Oxford: The Clarendon Press.

Warren, C. (1959). *J. biol. Chem.* **234**, 1971.

Webber, R. V. & Bayley, S. T. (1956). *Canad. J. Biochem. Physiol.* **134**, 993.

Yemm, E. W. & Willis, A. J. (1954). *Biochem. J.* **57**, 508.

Zimm, B. H. (1948). *J. chem. Phys.* **16**, 1093.