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The Protein–Polysaccharide Complex of Bovine Nasal Cartilage

STUDIES ON THE PROTEIN CORE

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1. Chondromucoprotein from bovine nasal cartilage was purified by cetylpyridinium chloride or by bismuth nitrate in acetone. 2. Amino acid compositions of crude and purified preparations were compared and few differences were found, in spite of the decrease in protein content on purification. 3. Amino acid analysis of bismuth-purified material revealed the existence of four groups of amino acids. Within each group, the amino acids were present in approximately equimolar concentrations. 4. Amino end-group assay on the same material showed six α-DNP derivatives. 5. A molecular weight of $6{\cdot}3 \times 10^5$ for the protein–polysaccharide complex was calculated from the latter analysis.

The macromolecular structure of the protein–polysaccharide complex of cartilage has been extensively studied since Schatton & Schubert (1954) showed that aqueous extracts of bovine nasal cartilage contained a high-molecular-weight component consisting of chondroitin sulphate apparently bound to a non-collagenous protein.

Webber & Bayley (1956) estimated, by sedimentation and viscosity methods, that the molecular weight of calcium chloride-extracted chondromucoprotein was 1×10^6. They also suggested that the complex had a random coil configuration and that it consisted of some 20 chondroitin sulphate molecules, of molecular weight approx. 4×10^4, linked by polypeptide chains in an end-to-end arrangement. Bernardi (1957*a*,*b*), working with a similar preparation, confirmed the random coil configuration and reported a molecular weight of $1{\cdot}98 \times 10^6$. Mathews & Lozaityte (1958) pointed out that light-scattering and viscosity data supported the existence of a rod-like fundamental molecular unit of molecular weight 4×10^6. They proposed a comb-like model in which the protein moiety was visualized as a core, 3700 Å in length, along which 62 chondroitin sulphate chains were uniformly distributed. This hypothesis of a continuous protein core was fundamentally confirmed by a study of the kinetics of degradation by hyaluronidase and papain of a preparation of protein–polysaccharide (Cessi & Bernardi, 1965).

Serafini-Fracassini & Smith (1966) examined by electron microscopy the protein–polysaccharide complex after precipitation of chondromucoprotein with bismuth nitrate in acetone. The precipitate appeared as rows of 20–25 dense particles. The length of the rows varied from 1100 Å to 1500 Å, and the particles had an average diameter of 30 Å. Each row was interpreted as being a protein–polysaccharide macromolecule and it was considered that each particle represented an individual chondroitin sulphate chain, in coil configuration, and the intervals between the particles represented the unstained protein core. This electron-microscopic appearance of the complex seems to be in keeping with the model proposed by Mathews & Lozaityte (1958), especially with respect to the distance between adjacent chondroitin sulphate chains; but there is disagreement about the length of the macromolecule. However, one of the preparations reported by Mathews & Lozaityte (1958) had a molecular weight, from light-scattering determinations, of $1{\cdot}7 \times 10^6$ and a mean length of 2900 Å. This length was calculated from the root-mean-square radius of gyration. In a polydisperse system of rods, the molecular species to which this size refers are those larger than the *z*-average (Ehrlich & Doty, 1954). Moreover, some contribution to length could be due to the polysaccharide. It is likely therefore that the number-average length would be closer to the range indicated by the electron-microscopic study of Serafini-Fracassini & Smith (1966).

It is generally agreed that various preparations of chondromucoprotein exhibit widely varying molecular weights (Webber & Bayley, 1956; Bernardi, 1957*a*,*b*; Mathews & Lozaityte, 1958; Partridge, Davis & Adair, 1961; Luscombe & Phelps, 1967). Mathews & Lozaityte (1958) pointed out that chondroitin sulphate–protein molecules may form aggregates of molecular weight up to 5×10^7 by

lateral and end-to-end association in which additional non-collagenous protein probably participates. On the other hand, Meyer (1966) suggested that the crude complexes of molecular weight above 1×10^6 and high protein content could be made up of smaller complexes bridged by basic proteins. This hypothesis was supported by the observation that chondromucoprotein preparations purified by chromatography on DEAE-cellulose have been reported to have a protein content of approx. 7·5% (Partridge, Whiting & Davis, 1965) and a molecular weight as low as $2{\cdot}4 \times 10^5$ (Partridge, 1966). Similar results were obtained by Buddecke, Kröz & Lanka (1963), who, by purification with cetylpyridinium chloride, obtained a material of molecular weight $5{\cdot}5 \times 10^5$, but characterized by a protein content of approx. 16%. The last-named authors also showed that, in the presence of Cu^{2+}, Ca^{2+} or Co^{2+}, molecular aggregates of molecular weight up to several million formed.

Finally, Mashburn, Hoffman, Anderson & Meyer (1965) reported that even the mildest methods of extraction yield a complex of sufficient electrophoretic inhomogeneity to prevent the definition of a discrete protein–polysaccharide complex. Similarly, Rosenberg, Johnson & Schubert (1965) showed that chondromucoprotein PP-L* from human costal cartilage can be separated into three fractions by the use of La^{3+}. Loewi (1964) and Loewi & Muir (1965) indicated that porcine chondromucoprotein could be separated by electrophoresis into immunologically distinct constituents.

This paper reports the composition, characterization and amino end-group analysis of the bismuth-precipitated protein–polysaccharide complex, the electron-microscopic appearance of which has been previously discussed (Serafini-Fracassini & Smith, 1966). The work is concerned mainly with the problem of the homogeneity of the complex with respect to its protein core.

MATERIALS AND METHODS

Extraction and purification of chondroitin sulphate–protein complex. Fresh bovine nasal septa were cleaned, planed into strips and extracted by the procedure of Malawista & Schubert (1958). Chondromucoprotein was fractionated by high-speed centrifugation into PP-L and PP-H by the method of Gerber, Franklin & Schubert (1960), all manipulations being carried out as quickly as possible at 4° to avoid proteolytic degradation. PP-L was precipitated from its solution in 0·15 M-KCl by addition of 2 vol. of ethanol. This material is referred to, in the present paper, as crude PP-L (PP-L-C).

For the preparation of the bismuth-purified PP-L (PP-L-Bi), a procedure was followed based on the electron-microscope staining technique described by Serafini-Fracassini & Smith (1966). The protein–polysaccharide complex was precipitated from a 2% (w/v) solution of PP-L-C in 0·15 M-KCl by slow addition of 4 vol. of $Bi(NO_3)_3$ in acetone [1 g. of $Bi(NO_3)_3$ was dissolved in 10 ml. of 2 N-HNO_3 and this was made up to 200 ml. with acetone]. The flocculent white precipitate was collected by centrifugation and washed with acetone and with distilled water. The precipitate was then stirred with 0·25 M-KCl for 3 hr. at 4°. Insoluble inorganic material was discarded after centrifugation and 2 vol. of ethanol was added to the supernatant, which was kept overnight at 4°, when a fine precipitate formed. This precipitate was collected by centrifugation, dissolved in 0·5 M-KCl, clarified by centrifugation and re-precipitated at 4° by the addition of ethanol. Finally, PP-L-Bi was dissolved in 0·15 M-KCl and centrifuged at $78000 g_{av.}$ for 30 min. The clear supernatant was exhaustively dialysed against distilled water at 4° and freeze-dried. The average yield was 80% of the starting material.

Precipitation of PP-L-C by cetylpyridinium chloride on a cellulose column followed by fractional elution of the complex with a salt gradient was adopted as an alternative purification procedure. The product was cetylpyridinium chloride-purified PP-L (PP-L-CPC). The method used was that of Scott (1960), as modified by Antonopoulos, Borelius, Gardell, Hamnström & Scott (1961). A linear gradient of $MgCl_2$ in 0·05% (w/v) cetylpyridinium chloride was used for the elution of the column. The eluate was monitored for its E_{260} by a base-compensating automatic recorder, and for its polyanion content by the turbidimetric method of Scott (1960). PP-L-CPC, eluted as a symmetrical peak, was separated from cetylpyridinium chloride by cooling and filtration (Scott, 1960). The purified protein–polysaccharide complex was precipitated by ethanol, dialysed against distilled water and then freeze-dried. The average yield was 75% of the starting material.

Assay of proteolytic activity of protein–polysaccharide preparations. The proteolytic activity of various PP-L preparations was tested according to the method described by Anson (1938), by using as substrate bovine haemoglobin that had previously been exhaustively dialysed against distilled water and freeze-dried. Citrate–phosphate buffers were used over the range pH 2–8.

Chemical determinations. Ash and moisture were estimated as described by Eastoe & Courts (1963).

Total nitrogen was estimated by the method of Chibnall, Rees & Williams (1943).

For hexosamines, hydrolysis was carried out by heating the sample in a sealed tube under N_2 with 4 N-HCl (2 ml. of acid/mg. of material) at 105° for 8 hr. After hydrolysis, excess of acid was removed in a rotary film evaporator at 30°.

Total hexosamine was estimated by the method of Cessi & Piliego (1960). Controls were run to allow for losses occurring during both hydrolysis and removal of acid.

Ogston (1964) pointed out that estimation of hexosamine in complex polysaccharides may give low results because other substances present may be converted, during hydrolysis, into products that subsequently decrease the yield of chromogen in the reaction with acetylacetone. To check this possible source of error, samples of bovine haemoglobin, ossein gelatin and serum albumin were hydrolysed under the conditions specified for hexosamine estimation. The

* Abbreviations: PP-L and PP-H, light fraction and heavy fraction respectively of chondromucoprotein.

recovery of known amounts of galactosamine added to these hydrolysates was then determined. The results showed that haemoglobin did not interfere even in 1000-fold excess. Gelatin lowered the yield if present in greater amount than 200-fold excess, but albumin, in the range 200–2000-fold excess, had the opposite effect. None of the proteins tested caused any significant interference in the protein concentration range of PP-L.

The two hexosamines were separated by the method of Partridge & Elsden (1961).

Thiol group was estimated by the method of Ellman (1959).

Amino acid analysis. Solutions of various PP-L preparations, in 200 times their own weight of constant-boiling HCl, were heated in sealed tubes under N_2 at 110° for 24hr. Additional hydrolyses were carried out for 36 and 72hr. with sample 1 and sample 5 (Table 1). Excess of acid was removed from the hydrolysates in a rotary film evaporator, the temperature of the water bath being maintained at 30°.

Alkaline hydrolysis for tryptophan was carried out by the procedure of Brenner, Niederwieser & Pataki (1965).

Amino acid analyses were carried out with a Technicon Auto-Analyser. Since the samples contained large amounts of hexosamines, it was found necessary to change the buffer gradient to avoid galactosamine merging with valine. This was done by lowering the pH of the first buffer from 2·875 to 2·750 and by adding to the first two chambers of the Autograd 5ml. and 3ml. respectively of methanol.

Amino end-group analysis. Samples of approx. 200mg. of dried protein–polysaccharide were dissolved in 50ml. of 0·15M-KCl in the reaction vessel of a Radiometer pH-stat set at pH 8·4. The microsyringe was filled with 0·25N-NaOH and N_2 was bubbled through the solution. After the pH had been stabilized, a 5ml. volume of a freshly prepared 5% (v/v) solution of 1-fluoro-2,4-dinitrobenzene in ethanol was added. The reaction was followed on the recorder and stopped when the rate of titration had levelled off. The viscous product was then exhaustively dialysed against distilled water at 4°. The dinitrophenylated material (DNP-PP-L) was collected by freeze-drying as it was soluble even on acidification of the medium and was not precipitated by the addition of 10vol. of acetone. Acid hydrolysis of DNP-PP-L for 12hr. at 110° in a sealed tube with a 200-fold excess of constant-boiling HCl gave rise to marked humin formation. Attempts to separate the DNP-amino acids from the various artifacts by chromatography on a silicic acid column (Steven & Tristram, 1962) were only partially successful and losses occurred. Resin hydrolysis was therefore adopted. The procedure was essentially that described by Steven (1962). Samples (200mg.) of DNP-PP-L were mixed with 30ml. of a thick aqueous slurry of Dowex 50 (X8; H^+ form; 200–400 mesh) in sealed tubes and heated for 30hr. at 100° in an oven fitted with a device for rotating the tubes about their mid-point at 10rev./min. α-DNP-amino acids were recovered from the resin by elution with boiling water. Amino acids and the remaining DNP-derivatives were then eluted with 0·8N-NH_3 soln.

Samples of the ether-soluble DNP-amino acids were subjected to two-dimensional thin-layer chromatography according to Brenner *et al.* (1965) on 20cm.×20cm. glass plates. The plates were developed with toluene–pyridine–2-chloroethanol–0·8N-NH_3 soln. (10:3:6:6, by vol.) in the first dimension and chloroform–benzyl alcohol–acetic acid (70:30:3, by vol.) in the second dimension. Samples of the water-soluble DNP-amino acids and the remaining amino acids were separated by thin-layer chromatography with butan-1-ol–acetic acid–water (4:1:1, by vol.) in the first dimension and phenol–water (3:1, w/v) in the second. The plates after development were sprayed with 0·3% ninhydrin in butan-1-ol acidified with acetic acid. Both the resin hydrolysis products of DNP-PP-L were rehydrolysed with constant-boiling HCl for 18hr. at 105°, in sealed tubes, and rechromatographed. This procedure was followed because, with resin hydrolysis, breakdown of protein to its constituent amino acids may not be complete and the presence of peptides could lead to misleading interpretation of chromatograms.

After development, the spots of the ether-soluble DNP-amino acids were removed according to the vacuum technique of Ritter & Meyer (1962). The DNP-amino acids were eluted from the 'thimbles' with chloroform–acetic acid (99:1, v/v) and E_{360} was measured. Quantitative thin-layer chromatograms were run in quadruplicate and samples of standard DNP-amino acids were also subjected to hydrolysis and chromatography to allow correction for losses.

Analysis of the acetone supernatant after precipitation of PP-L-Bi. The acetone supernatant was examined for free amino acids and peptides after PP-L-Bi had been collected by centrifugation. Acetone was removed in a rotary film evaporator; the residue was then neutralized with NaOH and extracted twice with butan-1-ol previously equilibrated with N-HCl. The extracts were evaporated to dryness and then desalted on a column of Dowex 50 (X8; H^+ form; 200–400 mesh) (Smith, 1960). The eluate from the column was evaporated to dryness, dissolved in a small volume of 80% (v/v) acetone and subjected to thin-layer chromatography with the solvent systems described for the analysis of the water-soluble DNP derivatives. The developed plates were sprayed with both acidified ninhydrin in butan-1-ol and Morgan–Elson reagent (Waldi, 1965).

Physicochemical measurements. The various PP-L preparations were dissolved in, and dialysed against, 0·15M-KCl before analyses.

A Spinco model E analytical ultracentrifuge with a schlieren optical system was used for ultracentrifugation. The constant-temperature control was set at 20°.

Partial specific volume was calculated from density measurements performed at 20° with two 10ml. pycnometers by the procedure of Washburn & Smith (1934).

Viscosity measurements were made at 20° with a capillary viscometer constructed as described by Schachman (1957).

RESULTS

Enzymic activity of PP-L preparations. Contamination of PP-L by proteolytic enzymes was assayed over a wide range of pH values. With PP-L-C two optima were found, at pH 2·5 and 7·5, with enzymic activities (expressed as m-equiv. of tyrosine liberated/hr./g. of PP-L) $9·0\times10^{-4}$ and $6·0\times10^{-4}$ respectively.

No proteolysis was detectable with PP-L-CPC and PP-L-Bi.

Protein content and composition of PP-L-C, PP-L-CPC and PP-L-Bi. To determine the protein content of the various PP-L preparations and the

Table 1. *Amino acid analysis of PP-L-Bi (sample 5)*

(A) Wt. of anhydro-amino acid (μg./10mg. of PP-L-Bi, ash- and moisture-free); (B) wt. of anhydro-amino acid (g./100g. of the protein component); (C) recovery after 24hr. hydrolysis (%).

Time of hydrolysis (hr.) ... ... Amino acid	(A) 24	(A) 36	(A) 72	(B) 24	(B) 36	(B) 72	Corrected values	(C)
Hydroxyproline	0·00	0·00	0·00	0·00	0·00	0·00		
Aspartic acid	121·59	116·50	99·79	8·58	8·22	7·04	9·36*	91·7
Threonine	74·81	71·47	55·70	5·30	5·04	3·93	6·08*	87·2
Serine	87·32	73·00	69·28	6·16	5·15	4·89	7·88†	78·3
Glutamic acid	190·19	201·38	166·57	13·42	14·21	11·75	14·21‡	94·4
Proline	116·95	107·22	88·22	8·25	7·57	6·23	9·16*	90·1
Glycine	77·88	78·38	57·87	5·50	5·53	4·08	5·53‡	99·3
Alanine	61·16	61·32	46·21	4·32	4·33	3·26	4·33‡	99·8
Valine	83·87	84·26	80·91	5·92	5·95	5·71	5·95‡	99·5
Cystine (half)	0·00	0·00	0·00	0·00	0·00	0·00	0·00	(68·8)§
Methionine	0·00	0·00	0·00	0·00	0·00	0·00	0·00	(45·4)§
Isoleucine	63·80	60·59	59·83	4·50	4·28	4·22	4·52*	99·5
Leucine	129·69	126·40	101·07	9·15	8·92	7·13	9·27†	98·7
Tyrosine	47·55	33·09	15·06	3·36	2·34	1·06	4·24*	79·3
Phenylalanine	63·45	70·85	54·17	4·48	5·00	3·82	5·00‡	89·4
Hydroxylysine	0·00	0·00	0·00	0·00	0·00	0·00	0·00	
Lysine	57·48	57·09	43·46	4·06	4·03	3·07	4·15†	97·8
Histidine	31·36	31·17	24·55	2·21	2·20	1·73	2·25†	98·2
Arginine	71·96	70·38	67·33	5·08	4·97	4·75	5·18*	98·0
Tryptophan	0·00	0·00	0·00	0·00	0·00	0·00	0·00	
Totals	1279·06	1243·10	1030·02	90·29	87·74	72·67	97·11	

Protein content extrapolated to zero time: 14·17%

* Extrapolation to zero time (24hr., 36hr. and 72hr. hydrolysates).
† Extrapolation to zero time (24hr. and 36hr. hydrolysates).
‡ Greatest value.
§ Calculated from amino acid analyses of sample 1.

individual amino acid recoveries after acid hydrolysis, one sample of PP-L-C (sample 1) and one of PP-L-Bi (sample 5) were analysed after hydrolysis for 24, 36 and 72hr. at 110°.

The compositions of the three PP-L-Bi (sample 5) hydrolysates are reported in Table 1, column (A), where the results are expressed as μg. of anhydro-amino acids/10mg. of dry and ash-free material. A corrected protein content of 14·17% was derived by extrapolation to zero hydrolysis time of the summated amino acid contents. The same calculation procedure was applied to the analytical results of sample 1, and a corrected protein content of 20·12% was obtained. Since the extrapolation curves of sample 1 and sample 5 had identical slopes and in both cases the percentage of zero-time recovery after 24hr. hydrolysis was 90·2, a correction coefficient of 1·108 was used for all other samples examined in the calculation of their corrected protein contents from the amino acid compositions of their 24hr. hydrolysates.

The amino acid composition values of the three PP-L-Bi hydrolysates converted into g. of anhydro-amino acids/100g. of the protein component are reported in Table 1, column (B). These values were used to estimate the influence of hydrolysis conditions on the individual amino acids. Glutamic acid, glycine, alanine, valine and phenylalanine were difficult to liberate, giving maximal values after 36hr. hydrolysis. Hydrolytic destruction of all other amino acids was apparent. Corrected concentrations of all these amino acids except four were determined by extrapolating to zero time their concentrations in all three hydrolysates, assuming a first-order reaction over the full range of hydrolysis as suggested by the linearity of their losses. Since a non-linear destruction of serine, leucine, lysine and histidine was found, corrected concentrations of these four amino acids were calculated by extrapolation, too, but by using the 24hr. and 36hr. hydrolysates only. For each amino acid, the percentage of zero-time recovery after 24hr. hydrolysis was calculated and reported in Table 1, column (C). Again good agreement was found with the recoveries calculated from the analytical data of sample 1.

Table 2. *Analyses of PP-L-C, PP-L-CPC and PP-L-Bi*

Values are calculated as percentages of dry ash-free samples.

	PP-L-C			PP-L-CPC	PP-L-Bi		
Sample	1	2	3	4	5	1	5+1
Protein	20·12	18·18	17·48	15·96	14·17	14·14	
Nitrogen	—	5·8	—	5·8	5·6	—	
Total hexosamine (as free base)	—	29·3	—	30·0	30·5	—	
Galactosamine: glucosamine ratio	—	9·6	—	10·6	10·5	—	

Amino acid composition of the protein component

Amino acid	Wt. of anhydro-amino acid (g./100g. of protein)						Amino acid residues (moles/1000 moles)
Hydroxyproline	0·00	0·00	0·00	0·00	0·00	0·00	
Aspartic acid	9·98	10·34	10·19	10·01	9·36	9·71	89·96
Threonine	5·46	5·45	5·58	5·56	6·08	5·55	62·45
Serine	7·11	7·31	6·81	7·46	7·88	7·87	98·19
Glutamic acid	14·59	15·21	15·23	15·93	14·21	14·29	119·83
Proline	9·32	7·82	9·11	9·14	9·16	9·23	102·80
Glycine	6·90	5·51	5·82	5·61	5·53	6·10	110·63
Alanine	5·00	5·37	4·64	4·99	4·33	4·36	66·37
Valine	5·93	6·88	6·06	5·74	5·95	5·90	64·89
Cystine (half)	1·22	Trace	Trace	0·00	0·00	0·00	
Methionine	1·54	0·00	0·00	0·00	0·00	0·00	
Isoleucine	3·95	4·87	4·92	4·50	4·52	4·80	44·70
Leucine	8·35	9·54	9·98	9·05	9·27	9·43	89·71
Tyrosine	4·16	4·38	4·70	4·20	4·24	3·67	26·32
Phenylalanine	4·43	4·94	6·36	5·61	5·00	4·98	36·81
Hydroxylysine	0·00	0·00	0·00	0·00	0·00	0·00	
Lysine	4·02	4·94	5·43	4·31	4·15	3·82	33·76
Histidine	2·19	2·11	2·26	2·22	2·25	2·22	17·69
Arginine	7·12	5·59	6·86	6·21	5·18	4·93	35·14
Tryptophan	Trace	—	—	0·00	0·00	0·00	

The extrapolated protein contents and the corrected amino acid compositions of the samples that have been analysed are reported in Table 2.

Traces of tryptophan have been found, after alkaline hydrolysis, only in sample 1 (PP-L-C).

Cysteine was estimated separately, but no significant amounts were found: less than 10^{-10} mole of SH groups/mg. of PP-L.

The values for nitrogen and hexosamine contents are reported in Table 2.

Amino end-group analysis of PP-L-Bi and PP-L-C. The results of the quantitative analysis of the *N*-terminal amino acids of PP-L-Bi are reported in Table 3 as moles/10^6g. of protein–polysaccharide. Acid rehydrolysis confirmed that the DNP derivatives had all been released by the resin hydrolysis.

No chromatographic evidence was obtained, either before or after the second hydrolysis, for the presence of *O*-DNP-tyrosine.

When samples of PP-L-C were examined, a considerable increase in the number of α-DNP-amino acids was found and the total amount of these was approximately twice that of the α-DNP-amino acids of PP-L-Bi. The crude preparation was found to contain in addition to the DNP derivatives reported in Table 3 the following: DNP-glutamic acid, DNP-alanine, DNP-phenylalanine and DNP-isoleucine.

Table 3. *End-group analysis of PP-L-Bi*

Values are expressed as moles of amino acid/10^6g. of protein–polysaccharide.

Aspartic acid	0·157
Threonine	0·112
Serine	0·183
Glycine	0·055
Valine	0·490
Leucine	0·592
Total	1·589

Analysis of the acetone supernatant. Chromatographic analyses carried out on the organic material liberated from PP-L-C by acetone at low pH

revealed the presence of peptides, hexosamine and the following free amino acids: isoleucine, phenylalanine, tyrosine, arginine, valine, alanine, glycine, glutamic acid, leucine and aspartic acid.

Sedimentation coefficients of PP-L-CPC and PP-L-Bi. The sedimentation coefficients at infinite dilution, $S^0_{20,w}$, of PP-L-CPC and PP-L-Bi, obtained by linear extrapolation of $1/S$ or $S(\eta/\eta_0)$ against c to $c=0$ by the method of least squares, were 12·41s and 12·34s respectively. The determination of $\bar{v}_{20}$ gave an average value of 0·686ml./g. for both preparations.

DISCUSSION

The protein content of PP-L decreases from an average value of 18·6% to 14·1% on purification of the protein–polysaccharide complex by precipitation with bismuth nitrate in acetone. The alternative purification by precipitation with cetylpyridinium chloride and selective resolubilization of the complex produces a material of intermediate protein content (16·0%). However, as shown in Table 2, the composition of the protein moiety changes very little on purification, the major features being a lower arginine content and the absence of tryptophan, methionine and cystine in both purified protein–polysaccharide complexes. Some of the slight differences in amino acid composition that are seen in different samples and preparations can be attributed to the contamination of PP-L-C by free amino acids and peptides that are removed by acetone at low pH, and by trace amounts of at least two proteolytic enzymes. Steven & Tristram (1962) pointed out that acetone extraction releases free amino acids and peptides from highly purified collagen. The presence of these compounds might therefore be considered as a physiological feature of connective tissues.

Even taking into account the difficulties of making deductions from overall amino acid analyses, the basic similarity in amino acid composition of crude and purified preparations, in spite of the decrease in protein content with purification, suggests that a proportion of the protein–polysaccharide macromolecules in PP-L-C may have a decreased chondroitin sulphate content. The latter could be naturally occurring degradation products of metabolic turnover in which some of the chondroitin sulphate chains have been cleaved from the protein core and then excluded from the domain of the macromolecule.

Examination of the amino acid composition of PP-L-Bi, expressed as residues/1000 residues (Table 2), reveals the existence of several groups in which the amino acids are present in approximately equimolar concentration. Serine, proline and glycine each appear to occur with the frequency of 1 in 10 residues; aspartic acid and leucine as 1 in 12 residues; threonine, alanine and valine as 1 in 16 residues and, finally, phenylalanine, lysine and arginine as 1 in 29 residues. Equimolarity of serine, glutamic acid, proline and glycine was reported by Anderson, Hoffman & Meyer (1965) in chondroitin 4-sulphate preparations obtained by proteolytic digestion of chondromucoprotein from nasal cartilage and characterized by an amino acid content of approx. 3%. A similar pattern was found by the same authors in samples of chondroitin 6-sulphate obtained from shark cartilage. But when DNP derivatives of the latter preparation were examined, several amino acids appeared to be *N*-terminal, indicating inhomogeneity of the small peptide chains.

These data suggest that the protein core of PP-L may be made up from a limited number of peptide 'sub-units' of slightly differing amino acid composition and sequence, but with the same amino acids involved in the linkage region with the polysaccharide.

The amino end-group analysis carried out on PP-L-Bi (Table 3) shows that six amino acids can be consistently detected as α-DNP derivatives. The quantitative determination of their concentrations reveals that they are present as approximately integral molar multiples of glycine. These findings alone might suggest that PP-L is a mixture of protein–polysaccharide macromolecules differing in their protein backbones. But this hypothesis cannot explain the striking similarity in amino acid composition of chondromucoprotein preparations purified by different methods. An alternative interpretation could be that the protein core of PP-L is made up of peptide 'sub-units', the sequential arrangement of which in the macromolecule is not strictly determined. If this were so, each 'sub-unit' would occupy the external position in the macromolecule at the free amino end with a frequency proportional to its occurrence in the whole protein core.

If this second hypothesis is correct, the number-average molecular weight of the protein–polysaccharide macromolecule can be calculated from the total number of moles of *N*-terminal amino acids/10^6g. of PP-L. The value of $6{\cdot}3\times10^5$, so obtained, is in good agreement with that ($5{\cdot}5\times10^5$) reported by Buddecke *et al.* (1963) in their study on PP-L purified with cetylpyridinium chloride. The similarity between PP-L-Bi and PP-L-CPC is emphasized in the present work by the identical values for their $S^0_{20,w}$.

Since the protein content of PP-L-Bi is 14·1%, the molecular weight of the protein core can be calculated as $8{\cdot}9\times10^4$, leaving a total molecular weight of $5{\cdot}4\times10^5$ for the carbohydrate moiety. The chain weight of chondroitin 4-sulphate, isolated

by papain digestion, has been reported as ranging between $2{\cdot}2 \times 10^4$ and $2{\cdot}8 \times 10^4$ (Mathews, 1956; Partridge *et al.* 1961; Buddecke *et al.* 1963). Therefore some 20–24 chondroitin 4-sulphate chains should be present in the protein–polysaccharide complex. These findings are in remarkable agreement with the results of the electron-microscopic examination of PP-L-Bi, where 20–25 particles, interpreted as chondroitin sulphate chains, were visualized to be arranged in single rows, 1100–1500 Å long (Serafini-Fracassini & Smith, 1966).

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