Isolation and Properties of Chondroitin Sulphates from Bovine Heart Valves

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1. Several protein-polysaccharides were isolated from the soluble extracts of bovine heart valves by sedimentation equilibrium in a caesium chloride density gradient (Meyer, Preston & Lowther, 1969). 2. Compositional and structural studies indicated that the polysaccharide moiety was chondroitin sulphate. Differences in the protein content of the products were observed. There was no evidence suggesting the presence of keratan sulphate. 3. Sedimentation studies indicated that the molecular weights of the samples were between 4.2×10^4 and 6.5×10^4 . The results are discussed in terms of a basic model for the protein-polysaccharides of two polysaccharide chains linked by a protein of variable size.

It is generally agreed that most chondroitin sulphates do not occur in tissues as free polysaccharides, but are covalently linked to protein. The amount of this complexed protein found in isolated preparations may be dependent upon either the purification technique or the type of tissue studied, although complexes containing various amounts of protein have been isolated from several tissues including bovine nasal cartilage (Pal, Doganges & Schubert, 1966; Hoffman, Mashburn, Meyer & Bray, 1967), bovine nucleus pulposus (Rosenberg & Schubert, 1967), human costal cartilage (Rosenberg, Johnson & Schubert, 1965) and pig laryngeal cartilage (Muir & Jacobs, 1967). There has been much discussion as to the native state of these protein-polysaccharide complexes in terms of the molecular weight of a basic unit. Recent studies have indicated that the chondroitin sulphate-proteins of large molecular weight may arise through interaction of the basic unit with a glycoprotein (Hascall & Sajdera, 1969).

Many of the previous studies on chondroitin sulphates have been made on material isolated from cartilaginous tissues. Little attention has been devoted to similar material from other types of tissues. It is therefore of interest to contrast the properties of the polysaccharide extracted from bovine heart valves with that isolated from the dense cartilaginous tissues. In previous papers (Lowther, Toole & Meyer, 1967; Preston, 1968; Toole & Lowther, 1968; Meyer *et al.* 1969) the identification, extraction and characterization of some mucopolysaccharides from bovine heart valves were reported and it was apparent in these observations that the experimental procedures used were com-

paratively mild. In the present paper, the isolation of several chondroitin sulphate-protein complexes from bovine heart valves is described. Our studies indicate that these protein-polysaccharides are of low molecular weight as compared with material isolated from bovine nasal cartilage or bovine nucleus pulposus. A variation in protein content within the material is noted. This is discussed in terms of a basic structure for the complexes of two polysaccharide chains linked to a protein core of variable size.

EXPERIMENTAL

Endogenous proteolytic activity released during extraction procedure. The presence of endogenous tryptic activity in the extract, which would result in degradation of the protein-polysaccharides, was investigated by the standard technique of Kunitz (1947) with casein as a substrate. The digestion of the casein by α -chymotrypsin (Type II; Sigma Chemical Co., St Louis, Mo., U.S.A.) was used as a control.

A more specific study for proteolytic activity was made by incubating a [14 C]leucine-labelled protein-polysaccharide prepared from bovine nasal cartilage (1.50mg; specific radioactivity 1020 c.p.m./mg) in the 0.2M-NaCl extract (1.0ml) at 4°C for several days. After incubation, the mucopolysaccharide was precipitated with CTAB* and the supernatant collected by centrifugation.

The presence of radioactive material in the supernatant was measured by standard counting techniques in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The radioactivity of solutions was counted in a mixture of 0.5ml of aqueous sample and 15ml of

* Abbreviation: CTAB, cetyltrimethylammonium bromide.

scintillation mixture prepared as described by Bruno & Christian (1961).

Extraction of mucopolysaccharides from heart valves. The material soluble in 0.2M-NaCl was extracted as described by Meyer *et al.* (1969). The residue was re-extracted with an equal volume of 1M-NaCl-0.12M-NaHCO₃, pH6.8, overnight, and the supernatant collected by centrifugation as described by Lowther *et al.* (1967).

Fractionation of salt extracts. The 0.2M-NaCl extract was fractionated by caesium chloride density-gradient centrifugation as described by Meyer *et al.* (1969). Further purification of the chondroitin sulphate-containing fraction (their fraction B, mean density 1.76g/ml) was achieved by recentrifugation of the sample at an initial density of 1.75g/ml at 100000g in the no. 50 rotor of the Beckman-Spinco model L preparative ultracentrifuge. After 72h, the contents of the tubes were divided into nine fractions by means of a Beckman tube slicer. The fractions shown to contain the chondroitin sulphate were collected, pooled, dialysed against water to free them from CsCl and stored at -20°C.

The mucopolysaccharides present in the 1M-NaCl extract were isolated directly by precipitation with CTAB as described previously (Toole & Lowther, 1966; Scott, 1960). This extract has been shown to contain mainly chondroitin sulphate (Lowther *et al.* 1967). Fractionation of this material was achieved by caesium chloride density-gradient centrifugation at an initial density of 1.72g/ml at 100000g for 48h. The contents of the tube were divided into three 3ml fractions with mean densities of 1.67, 1.72 and 1.76g/ml. The removal of any extraneous non-covalently linked protein from the top fraction was achieved by re-centrifugation at a density of 1.50g/ml at 100000g for 24h. Each fraction was assayed for its protein and uronic acid content after dialysis against water to remove the CsCl.

Isolation of chondroitin sulphate from chondroitin sulphate-protein complexes. The protein was removed from the complex by digestion with proteolytic enzymes. An initial treatment of the sample with papain (Mann Research Laboratories, New York, N.Y., U.S.A.) in 0.1M-Na₂HPO₄-KH₂PO₄-5mM-EDTA-5mM-cysteine, pH6.5, at 65°C for 18h was followed by dialysis against 0.5M-KH₂PO₄ overnight, the pH adjusted to 7.5 with 2M-NaOH and the material incubated with pronase (B grade, lot 63807; Calbiochem, Los Angeles, Calif., U.S.A.) at 37°C for 2h. The digest was dialysed against 0.05M-NaCl and the chondroitin sulphate isolated by the precipitation technique with CTAB (Lowther *et al.* 1967).

Treatment with hyaluronidase. The digestion of samples of chondroitin sulphate by testicular hyaluronidase was performed as described by Meyer *et al.* (1969). With the 1M-NaCl extract, the digestion did not proceed to completion, only 50-70% of the material being degraded. However, dialysis of the digestion mixture against 0.1M-sodium acetate-0.1M-NaCl, pH5.0, at 37°C overnight, decreased the amount of undegraded material to about 10%.

Characterization of the neutral sugars in the protein-polysaccharide. A pronase digestion of the 0.2M-NaCl-extracted protein-polysaccharide was carried out as described by Yamauchi, Makino & Yamashina (1968). The digest was dialysed for 48h against several changes of 0.05M-NaCl and the polysaccharide isolated by precipita-

tion with CTAB and further purified by chromatography on ECTEOLA-cellulose as given by Lowther *et al.* (1967). The 1.0M-NaCl eluate was dialysed against several changes of 0.05M-tris-HCl buffer, pH8.0, and 30 units of a chondroitinase from *Proteus vulgaris* (Robinson & Dorfman, 1969) was added to the solution, which was incubated at room temperature (20°C) overnight. The completion of the digestion was checked by the turbidimetric method of Moretti & Whitehouse (1963) with Rivanol as precipitant. The digest was applied to a column (1cm×10cm) of Dowex 1(X2; salicylate form; 200-400 mesh) (Yamashina, Makino, Miyakoshi & Sakai, 1963) and the neutral sugars were removed by elution with water (30ml). The sugars were detected by the orcinol-H₂SO₄ method of Hewitt (1937).

A 50% (w/v) solution of trichloroacetic acid was added to the eluate to give a final concentration of 10% trichloroacetic acid. This treatment precipitated the chondroitinase and the salicylate ion which were removed by centrifugation. The excess of trichloroacetic acid and any remaining salicylic acid in the supernatant were removed by repeated extraction with ethyl ether. The neutral monosaccharides were liberated by hydrolysis under an atmosphere of N₂ at 100°C for 8h in 0.5M-H₂SO₄ and identified by g.l.c. of the trimethylsilyl ether derivatives as described by Sweeley, Bentley, Makita & Wells (1963).

For the identification of the neutral sugars of the 1M-NaCl extract, the protein-polysaccharide (5.2mg) was hydrolysed in 0.5M-H₂SO₄ (0.2ml) as described above and the hydrolysate passed through successive columns of Dowex 1(X2; acetate form) (0.9cm×1.0cm) and Dowex 50(X8; H⁺ form) (0.9cm×1.0cm) to remove inorganic ions and amino compounds. The columns were washed with 10vol. of water, the effluent and washings were evaporated to dryness and the neutral sugars identified by g.l.c. as described above.

Alkali lability of the protein-to-polysaccharide linkage. This was studied by using slight modifications of the hydrolysis procedure of Anderson, Hoffman & Meyer (1965). The protein-polysaccharide (600μg) was dissolved in water (20μl), 0.5M-NaOH (20μl) was added and the reaction allowed to proceed under N₂ for 16h at 4°C. After neutralization with 0.5M-HCl, the hydrolysate (30μl) was applied to Whatman 3MM chromatography paper and electrophoresis performed in 0.1M-sodium acetate-6M-urea, adjusted to pH5.36 with 0.1M-acetic acid, for 2.5h at a voltage gradient of 10mV/cm. The presence of the polysaccharide moiety was detected with 0.1% toluidine stain.

Reagents. CTAB was of laboratory-reagent grade and purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Casein was purchased as Casein (Hammarsten) from British Drug Houses Ltd. Analytical-grade reagents were used in all experiments.

Determination of protein and sulphate. The methods described by Meyer *et al.* (1969) were used for the protein and sulphate determinations.

Determination of hexose. This was determined by the anthrone method of Trevelyan & Harrison (1952) with D-glucose (analytical reagent; British Drug Houses Ltd.) as standard. Where necessary values were corrected for the presence of uronic acid.

Determination of hexosamine. The routine determinations were as described by Meyer *et al.* (1969). The nature

of the hexosamine was determined on an acid hydrolysate by the column technique of Gardell (1953) or with the amino acid analyser by using similar conditions to those used for amino acid analysis.

Determination of uronic acid. This was determined by the carbazole methods of (a) Bitter & Muir (1962) and (b) Dische (1947) with D-glucuronic acid (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) as standard. Differences in response to these assays were used as an indication of the presence or otherwise of iduronic acid in the polysaccharide material.

Amino acid analysis. Protein-polysaccharide samples (about 10mg) were hydrolysed in 6M-HCl in vacuum-sealed ampoules at 105°C for 24h. After hydrolysis the samples were evaporated to dryness *in vacuo* over NaOH pellets, and the amino acid content was analysed on a Hitachi KLA-2B amino acid analyser.

For the sample isolated from the 0.2M-NaCl extract, only the acidic and neutral amino acids were determined because of the limited amount of material available.

Determination of chondroitin sulphate isomers. The proportions of chondroitin 4-sulphate and chondroitin 6-sulphate in the samples were measured as described by Robinson & Dorfman (1969). The chondroitin sulphate samples from both the 0.2M-NaCl and the 1M-NaCl extractions were digested individually with a purified chondroitinase of *P. vulgaris*. The resultant disaccharides were separated on Whatman 3MM paper with butan-1-ol-acetic acid-pyridine-water (15:3:10:12, by vol.) as solvent. The chromatogram was dried and the disaccharides, detected by their strong absorption when irradiated with u.v. light, were eluted from the chromatogram with water and measured by their hexosamine content. The standard unsaturated disaccharides were kindly given by Dr H. C. Robinson of this department.

Determination of i.r. spectra. These were measured with a Perkin-Elmer model 521 spectrophotometer. Samples (1mg) were prepared in KBr discs.

Determination of optical rotation. The specific rotation was determined at 25°C and 589nm in a Perkin-Elmer model 141 polarimeter.

Analytical sedimentation. A standard solvent buffer of

0.1M-NaCl-0.12M-NaHCO₃, pH 6.8, was used. Each sample was dialysed for 2 days against several changes of buffer before the experiment.

All experiments were performed in a Spinco model E ultracentrifuge equipped with both schlieren and Rayleigh interference optics. The temperature was maintained at 20°C by use of the RTIC unit.

Velocity sedimentation was observed at 59780 rev./min in cells with a 1.2cm single-sector (Kel-F; Minnesota Mining and Manufacturing Co., St Paul, Minn., U.S.A.) centrepiece. Sedimentation equilibria to determine the molecular weights of the samples were performed in cells with a 1.2cm double-sector centrepiece as described by Yphantis (1964). Calculations of the various average molecular weights, both whole-cell and point values, were performed on a Control Data 3200 computer with a FORTRAN programme kindly supplied by Dr J. McD. Armstrong.

Partial specific volumes. Owing to the limited availability of the various samples a direct measurement of their partial specific volumes was not made. Values of this quantity for the samples were estimated from a knowledge of their composition and an assumption of the partial specific volumes for the protein and polysaccharide moieties of 0.74 and 0.52 ml/g respectively.

RESULTS

There was no evidence for the release of endogenous proteolytic activity during the extraction procedure. The standard technique described by Kunitz (1947) indicated that no digestion of the added casein had occurred within 4h at 37°C. The more specific study with the labelled protein-polysaccharide also indicated no release of ¹⁴C-containing peptides for periods of incubation at 4°C of up to 10 days.

Composition and nature of the sodium chloride extracts. The distribution of hexosamine within the tube after density-gradient centrifugation of the

Table 1. Chemical composition and optical rotation of the chondroitin sulphates from bovine heart valves

(a), Determined by the carbazole method of Bitter & Muir (1962); (b), determined by carbazole method of Dische (1947). $\bar{\rho}$, Mean density of fraction (g/ml). Compositions are expressed as % dry wt. Molar ratios to galactosamine are in parentheses.

	Fractions extracted with		Protein-free chondroitin sulphate	Fractions from 1M-NaCl extract		
	0.2M-NaCl	1M-NaCl		$\bar{\rho} = 1.67$	$\bar{\rho} = 1.72$	$\bar{\rho} = 1.76$
Uronic acid (free acid)						
a	31.8 (1.04)	22.7 (1.06)	34.2 (0.96)	23.7	24.5	28.5
b	32.1	19.8	29.0	24.7	24.7	28.0
Galactosamine (free base)	28.3* (1.00)	19.7 (1.00)	32.7† (1.00)	—	—	—
Glucosamine (free base)	1.2*	0.9	<0.1†	—	—	—
Sulphate	16.4 (1.08)	10.8 (1.01)	17.6 (1.00)	—	—	—
Protein	5.5	32.3	—	31.7	23.5	16.3
Optical rotation ($[\alpha]_D^{25}$)	-19.8°	-34.3°	-30.3°	—	—	—

* Estimated by routine determination and confirmed by measurement on amino acid analyser.

† Estimated by routine determination and confirmed by procedure of Gardell (1953).

0.2M-sodium chloride extract (fraction B of Meyer *et al.* 1969) at an initial density of 1.75g/ml indicated that the top two fractions contained a small amount of hyaluronic acid as judged by the presence of glucosamine; the chondroitin sulphate was found in the bottom half of the tube. The protein/uronic acid concentration ratio was found to be constant in the bottom five fractions, suggesting that the chondroitin sulphate is associated with a small amount of protein.

Earlier studies had identified the polysaccharides in the 1M-sodium chloride extract as mainly chondroitin sulphate (Lowther *et al.* 1967). The fractionation of this extract by density-gradient centrifugation at an initial density of 1.72g/ml was carried out to study the compositional homogeneity of the material. In contrast with the behaviour of the 0.2M-sodium chloride extract, the distribution of chondroitin sulphate between the three arbitrarily chosen 3ml fractions was reasonably uniform. The composition and other results for the various fractions are summarized in Table 1. Assuming the hexosamine was *N*-acetylated and allowing for the cation (Na^+) equivalence for all the negatively charged groups, about 90% of the dry weight of the various fractions can be accounted for. The molar equivalence between the glucuronic acid, galactosamine and sulphate content of the main fractions (Table 1, columns 2, 3 and 4) taken in conjunction with the observed susceptibility of these fractions to testicular hyaluronidase treatment suggested that the polysaccharide moiety was mainly chondroitin sulphate. The ratio of the values obtained in the Bitter & Muir (1962) and Dische (1947) carbazole methods for measuring uronic acid was found to be approx. 1.0 in all cases except for the 1M-sodium chloride extract material and its constituent polysaccharide (Table 1, columns 3 and 4).

An obvious feature of the composition of the various chondroitin sulphate fractions is the variation of protein content, ranging from 5.5 to 31.7%. The mode of the preparative procedure would imply that the variation in protein content does represent a true variation in the amount of covalently linked protein.

The digestion of the samples with the chondroitinase from *P. vulgaris* indicated a marked difference in composition with regard to the two isomeric chondroitin sulphates. In the low-salt extract the chondroitin 6-sulphate/chondroitin 4-sulphate ratio is 1.5 whereas in the high-salt extract it is 0.67. The relative values of the specific optical rotation (Table 1, columns, 2 3 and 4) are consistent with these findings; the low value for the 0.2M-sodium chloride extract indicates the presence of an excess of the chondroitin 6-sulphate isomer (Meyer, Davidson, Linker & Hoffman, 1956). The i.r. spectra of the samples revealed absorption bands

Table 2. *Amino acid composition of the 0.2M-NaCl- and 1.0M-NaCl-extracted chondroitin sulphate-proteins*

No corrections were applied for the destruction of amino acids that occurs during the hydrolysis.

	Composition (residues/1000 residues)	
	0.2M-NaCl extract*	1M-NaCl extract
Asp	74	100
Thr	82	92
Ser	133	99
Glu	130	135
Pro	80	75
Gly	98	71
Ala	67	67
Cys	—	8
Val	51	59
Met	—	10
Ile	36	37
Leu	70	74
Tyr	14	22
Phe	35	35
Lys	—	53
His	—	21
Arg	—	33

* Owing to limited amount of material available only the acidic and neutral amino acids were determined. This analysis is calculated assuming the undetermined amino acid residues accounted for 14.4% of the total, as observed for the analysis of the 1M-NaCl extract.

again in general agreement with these observations but an additional band at 712cm^{-1} was noted with the polysaccharide moiety from the 1M-sodium chloride extract. This band suggests the presence of dermatan sulphate within the sample (Mathews, 1958).

The amino acid analyses of the 0.2M- and 1M-sodium chloride-extracted material are given in Table 2. The compositions are similar to those found for various other protein-polysaccharides (Barrett, 1968; Schubert & Hamerman, 1968). The enzymic degradation of the 0.2M-sodium chloride-extracted protein-polysaccharide and subsequent identification of the neutral sugars indicated the presence of both galactose and xylose in a molar ratio of 2.2:1. The total hexose content, as determined by the anthrone method, is accounted for by the presence of these two sugars.

The electrophoretic behaviour of the 0.2M- and 1M-sodium chloride-extracted protein-polysaccharides indicated that the treatment with alkali resulted in an increase in the mobility of the polysaccharide moiety. Similar observations were made in a parallel study on material isolated from bovine nasal cartilage.

Ultracentrifuge studies. The results of these

Table 3. *Physical properties of chondroitin sulphate samples; details of experiments and other data*Abbreviations: P-P, protein polysaccharide; $\bar{\rho}$, mean density of fraction (g/ml).

	0.2M-NaCl extract	1M-NaCl extract	P-P from nucleus pulposus	P-P from nasal cartilage	Protein-free chondroitin sulphate	Fractions from 1M-NaCl extract		
						$\bar{\rho} = 1.67$	$\bar{\rho} = 1.72$	$\bar{\rho} = 1.76$
$s_{20,w}^0$ (S)	3.04	8.05	11.9	33.0	2.07	—	—	—
Sedimentation equilibria								
Initial concn. (mg/ml)	0.10	0.55	0.40	0.20	1.0	0.7	0.7	0.75
Column height (cm)	0.26	0.26	0.28	0.27	0.26	0.33	0.32	0.28
Speed (rev./min)	24630	20410	10589	4908	31410	20410	20410	20410
$10^{-3} \bar{M}_n$	46.0	84.2	151.0	—	21.0	64.6	63.7	55.0
$10^{-3} \bar{M}_w$	44.0	89.7	165.6	1238.1*	21.1	64.7	64.7	56.1
$10^{-3} \bar{M}_z$	49.0	113.0	229.5	—	22.0	62.2	62.5	55.0
$10^{-3} \times$ Mol. wt. of poly- saccharide moiety	42.0	—	—	—	—	44.0	46.0	47.0

* Solution displayed marked non-idealities, thereby invalidating estimates of $\bar{M}_n$ and $\bar{M}_z$.

experiments are summarized in Table 3. It is evident that the molecular weights of the chondroitin sulphate samples from bovine heart valves are lower than those found for similar material prepared from bovine nucleus pulposus and nasal cartilage (columns 4 and 5, Table 3). The molecular weight of the polysaccharide moiety (column 6, Table 3) is in general agreement with the various reported values for protein-free chondroitin sulphate (Barrett, 1968). An obvious feature of the results is the polydispersity of molecular weight observed for the unfractionated 1M-sodium chloride extract and for the preparations from the other two tissues. This is in marked contrast with the results for the 0.2M-sodium chloride extract and the fractionated 1M-sodium chloride extract. In all these cases (columns 2, 7, 8 and 9, Table 3) the sample appears to be monodisperse with regard to molecular weight. It is noteworthy that the polydispersity of molecular weight observed in the unfractionated 1M-sodium chloride extract cannot be explained by any marked differences in the molecular weights of the derived fractions.

DISCUSSION

The presence of endogenous hyaluronidases and proteases in various tissues have been reported (Bollet, Bonner & Nance, 1963; Tourtellotte, Campo & Dziewiatkowski, 1963; Reich & Buddecke, 1967) and studies *in vitro* (Luscombe & Phelps, 1967) indicate that such enzymes may depolymerize protein-polysaccharides. During the present investigation, several lines of evidence suggested that similar enzymes, if indeed they exist in the heart-valve tissue, are apparently not active and that the isolated materials are in fact native and not degradation products of larger molecules. High-molecular-weight hyaluronic acid similar to that

found in bovine synovial fluid has been isolated from the heart valve (Meyer *et al.* 1969), thereby indicating the absence of any hyaluronidase activity in the extract. This deduction is supported in the present study by the isolation of protein-free chondroitin sulphate chains, which were monodisperse with respect to molecular weight and of a similar size to material obtained from other much larger complexes. The complete absence of any proteolytic activity in the extracts strongly suggests that the low-molecular-weight protein-polysaccharides have not arisen by degradation of the protein component of some larger entity. It is also noteworthy that when the present isolation procedures were applied to other tissues, the chondroitin sulphates prepared were of much larger molecular weight (Table 3). These observations suggest that the small molecular size of the material from heart valves is not an artifact of the isolation technique but represents a true tissue variant.

The differences in the estimated uronic acid contents of the 1M-sodium chloride-extracted material and its polysaccharide moiety (Table 2), obtained by using the two carbazole methods, can be understood in terms of the presence of iduronic acid (Bitter & Muir, 1962). The disappearance of this discrepancy on the subsequent fractionation of the extract by density-gradient fractionation would suggest that the iduronic acid-containing material was removed during this process. The presence of dermatan sulphate, known to contain iduronic acid, in the polysaccharide isolated from the unfractionated 1M-sodium chloride extract is suggested from the i.r. spectra. The occurrence of dermatan sulphate would explain the observed partial resistance to hyaluronidase of this material. If the iduronic acid-containing material is similar in composition to the dermatan sulphate-protein shown to be

present in the heart-valve tissue (Toole & Lowther, 1965; Toole & Lowther, 1968), then its high protein content and consequent low buoyant density would result in this material floating on the top of the gradient and in its subsequent removal during the fractionation.

The behaviour of the salt extracts during caesium chloride density-gradient centrifugation is understood in terms of the chondroitin sulphate samples being complexed with various amounts of protein. The distribution of material in the gradient with the 0.2M-sodium chloride extract indicated the presence of a chondroitin sulphate containing a relatively constant amount of protein whereas a similar study with the 1M-sodium chloride extract suggested the presence of chondroitin sulphate linked to a variable amount of protein. The fractionation of this material yielded samples with different protein contents. The ultracentrifugation studies that indicate that these samples may represent discrete preparations, homogeneous with regard to molecular weight and perhaps composition, may be misleading. Any fractionation of material with a molecular weight spread of 55000–65000 would probably yield a sample appearing to be homogeneous within the accuracy of the sedimentation studies. A more likely situation is that the chondroitin sulphate exists linked to a continuously variable amount of protein. Calculations based on protein content and molecular weight of the samples suggest that the amount of chondroitin sulphate present in the samples is reasonably constant (bottom row, Table 3). This observation, taken in conjunction with the molecular weight of the protein-free chondroitin sulphate chains, would suggest a basic model for the protein–polysaccharide of two polysaccharide chains linked by a protein of variable size.

The analytical and compositional studies on the various fractions clearly indicate that the polysaccharide moiety is chondroitin sulphate. Unlike similar preparations from other tissues, there is no evidence for the presence of keratan sulphate. The neutral-sugar content and the alkali lability of the protein–polysaccharide linkage implicates a linkage region similar to that found for other connective-tissue polysaccharides (Roden & Smith, 1966) in which the polysaccharide chain is joined to the protein through the neutral sugars to the hydroxyl group of serine or threonine. It appears that the low-ionic-strength solvent extracts a chondroitin sulphate different from that isolated with 1M-sodium chloride both in protein content and in the isomeric form of the chondroitin sulphate. It is thought that these variations may reflect a difference in organization of the material within the tissue.

The biological role of protein–polysaccharides in various tissues has been suggested as controlling

the degree of hydration of the tissue and effecting the diffusional and bulk transport of solutes through the tissue (Laurent, 1966). These functions can be regarded as consequences of the large size and diffuse nature of the complex and also of the high concentration at which the material is found in the tissues. In our present studies, the low size and the low concentration of the protein–polysaccharides that are isolated from the heart valves preclude it from having a similar biological role. Preliminary evidence from studies *in vitro* does suggest that this material may be involved in collagen-fibre formation.

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