Studies on the Protein-Bound Chondroitin Sulphate of Bovine Cortical Bone

By G. M. HERRING

*Medical Research Council Bone-Seeking Isotopes Research Unit,
Churchill Hospital, Headington, Oxford*

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A fraction containing chondroitin sulphate, isolated from bovine cortical bone under mild conditions, was separated by ion-exchange chromatography into three fractions with apparent homogeneity on electrophoresis and ultracentrifugation. Two of these appeared to consist of chondroitin sulphate bound to a glycoprotein 'core' that had similarities to the bone sialoprotein described previously. The differences in composition of the two fractions were considered to be due to variation in the number or lengths of the polysaccharide chains. The presence of xylose and the alkali-lability of the bond between protein and polysaccharide suggested the presence of a xylosylserine linkage. The third fraction had the properties of a relatively pure chondroitin sulphate which contained a small amount of peptide. These fractions differed considerably from the protein-polysaccharide complexes of epiphyseal and other cartilages, and their relevance to the possible role of glycosaminoglycans is discussed.

Studies by Rogers (1949, 1951) indicated that the main glycosaminoglycan of cortical bone was a chondroitin sulphate. This was confirmed by Meyer, Davidson, Linker & Hoffman (1956) who found that decalcified bone contained 0.25% of chondroitin 4-sulphate, which was the only mucopolysaccharide detected. Experiments by Hjertquist & Vejlens (1966) on dog bone provided similar results; chondroitin 4-sulphate, characterized by its infrared spectrum, was isolated in a yield of 0.32–0.36% of organic matrix. However, a small amount of hyaluronic acid, about 3% of the total glycosaminoglycan, was also noted. In these studies proteolytic digestion was used to obtain maximum yields of polysaccharide. However, suitable extraction methods have shown that the chondroitin sulphate of epiphyseal cartilage (Castellani, Bonferoni, Ronchi, Ferri & Maleovati, 1962; Castellani, 1965; Castellani, Zonta & De Luca, 1965), and of other cartilages, occurs linked through covalent bonds to protein, so forming high-molecular-weight complexes (Shatton & Schubert, 1954; Malawista & Schubert, 1958; Muir, 1958; Partridge & Davis, 1958). It was therefore considered of interest to study the composition of the chondroitin sulphate isolated from bone by relatively mild procedures to see if similar types of protein-bound complexes also occur in this tissue. Information on such constituents may be of some importance when one is considering the suggested role of glycosamino-

glycans in the mechanism of calcification (see reviews by Weidmann, 1963; Balazs & Rogers, 1965).

A preliminary account of the present work has been published (Herring, 1967).

MATERIALS AND METHODS

The methods used for the preparation of powdered cortical bone, extraction with sodium EDTA solution and fractionation of the soluble constituents were as described previously (Herring, 1964). An outline of the fractionation procedure is shown in Scheme 1, which includes slight modifications to the original method: (a) the chondroitin sulphate fraction was precipitated with CPC* in 0.2M-MgCl₂ instead of 0.15M-MgCl₂, and (b) 2M- instead of 0.6M-MgCl₂ was used to dissolve the CPC-chondroitin sulphate complex, to ensure the maximum recovery of uronic acid-containing material.

Purification of fraction ED'. Precipitation with CPC was repeated three times. Fraction ED' (see Scheme 1) (500 mg.) was dissolved in 0.2M-MgCl₂ (20 ml.) and an excess of CPC solution (2%, w/v, in 0.2M-MgCl₂) added with stirring. After 1 hr. at room temperature, the precipitate was collected by centrifugation, and the supernatant solution was filtered through a thin pad of Celite (analytical filter aid; Johns-Manville and Co. Ltd., London, S.E. 1) on a sintered-glass funnel. The precipitate was washed with 30 ml. of 0.2M-MgCl₂ containing 0.1% (w/v) of CPC, and dissolved in 2M-MgCl₂ (25 ml.), which was then filtered through the Celite pad. Sufficient saturated KCNS was

* Abbreviation: CPC, cetylpyridinium chloride.

Scheme 1. Flow sheet illustrating the isolation of the chondroitin sulphate fraction from bovine cortical bone.

added to precipitate the CPC as the insoluble thiocyanate, which was removed by filtration through Celite. The filtrate and washings were dialysed against water (three changes of 1 l. for 24 hr.), concentrated, and precipitated by the addition of 4–5 vol. of ethanol containing potassium acetate (2%, w/v) and acetic acid (2%, v/v). The yield of fraction ED' was 54% of fraction ED'.

The final product (fraction ED'') from the above procedure was then subjected to precipitation with 9-aminoacridine hydrochloride by a method based on that described by Muir (1958). Fraction ED'' (500 mg.) was dissolved in water (25 ml.), and acetic acid was added until the solution was just acid to methyl orange. A saturated solution of 9-aminoacridine hydrochloride was added with stirring until the precipitate flocculated, and the solution was kept overnight at 4°. The precipitate was collected by centrifugation, washed three times with water (10 ml.) and redissolved by the addition of 20 ml. of 2 M-potassium acetate buffer, pH 5.0, containing 15% ethanol. The chondroitin sulphate was precipitated by the addition of 4 vol. of ethanol. After 16–20 hr. at 4°, the precipitate was collected by centrifugation and washed three times with ethanol.

The above precipitation procedure was repeated twice, and the final product, fraction ED''', was obtained in a yield of 87% of fraction ED''.

Electrophoresis. An Antweiler micro-electrophoresis apparatus was used for free-boundary electrophoresis experiments. Solutions (1–2%, w/v) of the preparations were used in 0.1 M-sodium barbitone-HCl, pH 8.6, or in 0.1 M-potassium acetate buffer, pH 5.0. A current of 1.35–1.65 mA and a voltage of 60–70 V produced a satisfactory

separation after 8–9 min. On completion of the run, concentration gradients through the cell were measured with the interferometer and the points for a gradient curve calculated from these readings.

Cellulose acetate electrophoresis was carried out in a Shandon Universal electrophoresis apparatus with 0.1 M-sodium barbitone-barbitone buffer, pH 8.6. Four cellulose acetate strips, 18 cm. × 5 cm. (Oxoid Ltd., London, E.C. 4), were mounted in the apparatus with a bridge width of 12 cm., and a satisfactory separation of the components was obtained after 2 hr. at 50 V and 3.5 mA.

The strips were dried at room temperature for 30 min. before being stained. The more acidic substances, including the sialoprotein and glycosaminoglycans, were demonstrated by staining with alcian blue [1% (w/v) in 1% (v/v) acetic acid] for 10 min. followed by washing in water. Ponceau S (Korotser, Bergquist & Searcy, 1961) and Nigrosin (Kohn, 1958) were used to detect proteins.

Ultracentrifugal analysis. Solutions containing 0.8 mg./ml. of preparations in 0.1 M-NaCl were sedimented at 59680 rev./min. in a Spinco model E ultracentrifuge. Photographs of the schlieren patterns were taken at intervals after the rotor had reached maximum speed.

CPC solubility curves. The solubility of the CPC complex of fraction ED''' in 0.1–1 M-MgCl₂ was studied by the method of Antonopoulos, Borelius, Gardell, Hamnström & Scott (1961).

Gel-filtration chromatography. A column (30 cm. × 2 cm.) of Sephadex G-200 (superfine grade) in M-NaCl was prepared. After equilibration, a sample of fraction ED''' (6 mg.) in M-NaCl (0.5 ml.) was applied, and the column was eluted with the same solution. Protein (Lowry, Rosebrough, Farr & Randall, 1951) and uronic acid (orcinol method) were determined on the fractions (2 ml.) that were collected. Blue dextran was used to determine the void volume of the column.

DEAE-cellulose chromatography. For analytical separations of 6–15 mg. of material, columns (9 cm. × 1 cm.) of DEAE-cellulose [Sigma (London) Chemical Co. Ltd., London, S.W. 6] in 0.05 M-potassium acetate buffer pH 5.0, were prepared. For smaller amounts of the fractions (~2 mg.) a column 5 cm. × 0.6 cm. was used. After equilibration of the columns by washing with buffer, samples of the fractions were applied in 0.05 M-acetate buffer, pH 5.0. Elution with a flow rate of about 10 ml./hr. was carried out either with a linear gradient of 0.5–1 M-NaCl in 0.05 M-acetate buffer, pH 5.0, or stepwise with increasing concentrations of NaCl in 0.05 M-acetate buffer. Phenylmercuric acetate (0.001%, w/v) was added to all buffers as a general bacteriostatic agent.

On a preparative scale, a column 19 cm. × 2 cm. was used for the separation of components from fraction ED''' by stepwise elution. Fractions (10 ml.) were collected and the elution of material from the column was followed by measurement of the extinction at 240 or 280 mμ.

Fractions from each peak were pooled, reduced in volume to 3–5 ml. by concentration-dialysis against Carbowax 20M (G. T. Gurr Ltd., London, S.W. 6) and dialysed against water (four changes of 500 ml. for 24 hr.), and the solute was precipitated by the addition of 4–5 vol. of ethanol containing potassium acetate (2%, w/v) and acetic acid (2%, v/v). Each component required two or three further separations under the same elution conditions before homogeneity on electrophoresis was apparent.

Analytical methods. Amino acids. Quantitative determination of amino acids was carried out on an automatic analyser (Evans Electroselenium Ltd., Halstead, Essex) by the methods of Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958). Samples were hydrolysed for 17 hr. in 2.5 ml. of constant-boiling HCl at 110° under nitrogen.

Protein. A semiquantitative estimation of total protein was made by the method of Lowry *et al.* (1951) with crystallized albumin as the standard.

Nitrogen. This was determined by the method of Jacobs (1962).

Uronic acid. This was determined by the carbazole method of Bitter & Muir (1962), and by the orcinol method (Brown, 1946) with a 90 min. heating period.

Amino sugars. For the determination of amino sugars, samples were hydrolysed in 4N-HCl for 16 hr. (Partridge & Davis, 1958) and the hydrolysates evaporated to dryness over NaOH pellets at 4°. The method of Kraan & Muir (1957) was used with the following modification (Johnston, Ogston & Stanier, 1951): before addition of the Ehrlich's reagent, the tubes were allowed to equilibrate at 37°, and after the addition they were kept at this temperature for 1 hr. before measurement of the extinction at 535 m μ . Glucosamine was used as the standard. Glucosamine and galactosamine were separated and determined by the method of Gardell (1953).

Hexose. Hexose was determined by the anthrone method of Yemm & Willis (1954) with galactose as a standard. Corrections for the presence of chondroitin sulphate were made with controls containing equimolar mixtures of glucuronic acid and galactosamine.

Sialic acid. This was determined by the method of Warren (1959) and the direct Ehrlich procedure (Werner & Odin, 1952) with *N*-acetylneuraminic acid as the standard.

Sulphate. This was determined by the method of Dodgson (1961).

Paper chromatography of neutral sugars. Samples containing about 100 μ g. of hexose were hydrolysed in 2 ml. of 2N-HCl for 2 hr. at 100°. The hydrolysates were neutralized by the addition of dry Dowex 1 (X8; HCO₃⁻ form; 100–200 mesh) (Hartree, 1964) and filtered. The filtrate and washings (10 ml.) were passed through a short column (2 cm. $\times$ 1 cm.) of Dowex 50 (X8; H⁺ form; 100–200 mesh) to remove cations and hexosamines, and the eluate was evaporated to dryness over P₂O₅ under reduced pressure at 4°. Samples containing 30–40 μ g. of hexose were chromatographed on Whatman no. 1 paper by descending elution with ethyl acetate–pyridine–water (8:2:1, by vol.) (Leopold, 1962) or with ethyl acetate–acetic acid–water (3:2:1, by vol.). The paper had previously been washed with 10% acetic acid and water. Reducing sugars were detected by the alkaline AgNO₃ method of Trevelyan, Procter & Harrison (1950).

RESULTS

The compositions of the fractions ED', ED'' and ED''' are shown in Table 1. Cellulose acetate electrophoresis demonstrated that after the first separation fraction ED' still contained the 'sialo-protein' but this was removed by the subsequent precipitations with CPC under the conditions

Table 1. *Composition of the protein-bound chondroitin sulphate fractions obtained at the three stages of the purification procedure*

Experimental details are given in the text. ED', crude fraction; ED'', after three precipitations with CPC; ED''', after three precipitations with 9-aminoacridine hydrochloride.

Fraction	Composition (% of dry wt.)		
	ED'	ED''	ED'''
Nitrogen	5.2	4.1	4.1
Protein	15.1	12.0	11.5
Uronic acid (by the carbazole method)	20.2	23.1	23.1
Hexosamine	18.4	21.2	20.8
Sulphate	12.0	13.4	13.9

stated. Fraction ED''', obtained by three reprecipitations with 9-aminoacridine, did not differ appreciably from fraction ED'' in composition or electrophoretic appearance. The final material (fraction ED''') used in the subsequent work thus contained considerable amounts of protein and contained at least two acidic electrophoretic components.

As a possible method for the separation of the components of fraction ED''', a CPC solubility curve was prepared. This resembled the curve obtained for purified chondroitin sulphate (Antonopoulos, Gardell, Szirmai & de Tyssonsk, 1964) except that slightly higher concentrations of magnesium chloride were required for complete solubilization. Since there were no appreciable inflexions in the curve, it was concluded that the components of fraction ED''' were closely similar with regard to their CPC solubility characteristics.

The elution pattern of fraction ED''' on gel-filtration chromatography indicated that the main part of the peak behaved as a relatively high-molecular-weight component, appearing shortly after the void volume. However, this might reflect molecular size rather than molecular weight, since an acidic polysaccharide might be expected to have a relatively expanded structure in solution compared with a protein molecule. The ratio of protein to uronic acid was higher (1.8 and 1.2) in the first two fractions than in the remainder of the peak, where the ratio varied only slightly (0.5–0.7), but no appreciable resolution of the electrophoretic components could be detected when fractions from different parts of the peak were examined by cellulose acetate electrophoresis. The apparent dispersity and trailing of the peak suggested some adsorption effects.

Chromatography on DEAE-cellulose. The behaviour of fraction ED''' on DEAE-cellulose at

Fig. 1. Chromatography of fraction ED''' (10.1 mg.) on a DEAE-cellulose column (10 cm. $\times$ 1 cm.). Elution was with a 0.05 M-acetate buffer, pH 5, and a linear gradient of 0.5–1.0 M-NaCl. Fractions (1 ml.) were collected. Uronic acid (by the orcinol method), —; protein (by the modified biuret method), ---.

Fig. 2. Chromatography of fraction ED''' (9.4 mg.) on a DEAE-cellulose column (10 cm. $\times$ 1 cm.). Elution was with a 0.05 M-acetate buffer, pH 5, and with 0.65 M-, 0.7 M- and 0.8 M-NaCl introduced at the points shown by the arrows. Fractions (1 ml.) were collected. Uronic acid (by the carbazole method), —; protein (by the modified biuret method), ---.

pH 5.0 with a gradient of 0.5–1.0 M-NaCl is shown in Fig. 1. Alterations in column size or elution conditions did not resolve the protein from the uronic acid peak. Samples taken from the earlier fractions in the peak contained the slower-moving and those taken from the later fractions contained the faster-moving electrophoretic components.

Further studies were made with stepwise elution, and it was found that no material was eluted with 0.6 M-NaCl, most of the protein was eluted with 0.7 M-NaCl and most of the uronic acid was eluted with 0.8 M-NaCl. With steps of 0.65 M-, 0.7 M- and

Fig. 3. Chromatography of separated fractions (a) D1 (5.9 mg.), (b) D2 (6.2 mg.) and (c) D3 (5.0 mg.) on a DEAE-cellulose column (10 cm. $\times$ 1 cm.). Elution was with a 0.05 M-acetate buffer, pH 5, and with 0.65 M-, 0.7 M- and 0.8 M-NaCl introduced at the points shown by the arrows. Fractions (2 ml.) were collected. Uronic acid (by the orcinol method), —; protein (by the modified biuret method), ---.

0.8 M-NaCl the elution pattern shown in Fig. 2 was obtained.

The fractions D1, D2 and D3 isolated on a preparative scale gave elution patterns as shown in Fig. 3. Fractions D1 and D3 gave single peaks with the previous elution conditions, but fraction D2 showed some material (probably fraction D1) eluted with 0.65 M-NaCl. These preparations were used for the subsequent studies. Cellulose acetate electrophoresis followed by staining with alcian blue gave the separations shown in Fig. 4; ultracentrifugal analysis is shown in Fig. 5 and the analytical results on the three fractions are shown in Tables 2 and 3.

Amino acid analyses were carried out on fraction

Fig. 4. Cellulose acetate electrophoresis of fraction ED''' and separated component fractions D1, D2 and D3. Sodium barbitone-barbitone buffer (0.1 M; pH 8.6) was used with a current of 3.5 mA (50 v) for 2 hr. The strips were stained with alcian blue.

Fig. 5. Ultracentrifuge patterns of separated fractions D1, D2 and D3 82 min. after reaching maximum speed (59680 rev./min.). (a) Upper trace, D2; lower trace, D1; (b) upper trace, D2; lower trace, D3. The direction of movement was from right to left.

D1 (4.048 mg.), fraction D2 (5.72 mg.) and fraction D3 (7.592 mg.) from which, respectively, 1.112, 1.245 and 0.136 mg. of total amino acids were obtained. Because of the small amount of protein analysed, the results for fraction D3 can only be regarded as semiquantitative. The value for lysine in fraction D3 (Table 3) appeared to be exceptional and was considered to be largely an artifact, since measurements in the ninhydrin reaction showed that the ratio of the extinction at 440 m μ to that at 570 m μ was 0.44 in the case of authentic lysine but was 0.25 for the 'lysine' peak in fraction D3. Artifacts, appearing near to lysine in amino acid separations, and assumed to be degradation products formed from galactosamine and uronic acid, have been reported to be present in hydrolysates of chondroitin sulphate (A. A. Castellani, personal communication; Anderson, Hoffman & Meyer, 1965; Muir & Jacobs, 1967). These might be expected to predominate in fraction D3, where the ratio of chondroitin sulphate to peptide is about 30 times the ratio in fractions D1 and D2. In Table 3 the values for fraction D3 are given in the third column as found, and in the fourth column corrected on the assumption that only 30 residues may be attributable to true lysine. On this basis the corrected total amino acid content of fraction D3 (Table 2) would be 1.27%.

Paper chromatography of the aldoses from hydrolysates of fraction D1 indicated the presence of galactose and mannose with smaller amounts of fucose and xylose in approximately equal proportions. In fraction D3, however, only galactose, with a possible trace of xylose, was observed.

Alkaline hydrolysis was carried out by the method of Partridge & Davis (1958). Fraction D1 (2.12 mg.) in 0.5 N-NaOH (2 ml.) was allowed to stand at room temperature for 16 hr. After neutralization and dialysis against 0.05 M-potassium acetate buffer, pH 5.0, the sample was applied to a column (5 cm. $\times$ 0.5 cm.) of DEAE-cellulose as

Table 2. *Composition of the protein-bound chondroitin sulphate fractions isolated by ion-exchange chromatography on DEAE-cellulose*

Fraction ...	Composition (% of dry wt.)		
	D1	D2	D3
Nitrogen	6.2	5.5	2.7
Protein (amino acids)	27.46	21.76	1.80
Uronic acid (by the carbazole method)	17.1	22.1	30.1
Uronic acid (by the orcinol method)	18.1	23.4	31.8
Galactosamine	13.9	17.9	25.1
Glucosamine	1.7	1.2	<0.1
Hexose	5.2	4.2	0.9
Sialic acid (by the direct Ehrlich method)	13.1	7.8	<0.2
Sialic acid (by the thiobarbituric acid method)	12.1	6.9	<0.05
Sulphate	8.5	10.9	16.9

Table 3. *Amino acid composition of the protein-bound chondroitin sulphate fractions isolated by ion-exchange chromatography on DEAE-cellulose*

The bone sialoprotein results are calculated from the results of Andrews & Herring (1965).

Amino acid	Fraction ...	Amino acid composition (residues/1000 total amino acid residues)					Bone sialoprotein
		D1	D2	D3	D3 'corrected'*		
Lysine		33	34	(283)	30		29
Histidine		24	20	36	49		13
Arginine		48	42	13	19		11
Aspartic acid		162	162	101	138		173
Threonine		75	87	59	81		111
Serine		111	97	82	111		80
Glutamic acid		200	194	96	131		228
Proline		61	63	61	83		49
Glycine		127	140	93	127		122
Alanine		40	38	53	73		40
Valine		29	31	19	26		30
Methionine		3	2	3	3		—
Isoleucine		15	19	24	33		25
Leucine		40	34	50	68		29
Tyrosine		15	19	9	12		22
Phenylalanine		15	16	14	20		11

* Values calculated on the basis of 30 residues of lysine; see the text for details.

Fig. 6. Chromatography of (a) fraction D1 (2.12mg.) and (b) alkali-treated fraction D1 (2.12mg.) on a DEAE-cellulose column (5cm. $\times$ 0.6cm.). Elution was with a 0.05M-acetate buffer, pH5, and a linear gradient of 0.2–0.8M-NaCl. Fractions (4ml.) were collected. Uronic acid (by the orcinol method) —; protein (by the modified biuret method) ---; sialic acid (by the direct Ehrlich method) - - -.

Fig. 7. Cellulose acetate electrophoresis of fraction D1 and alkali-treated fraction D1. Sodium barbitone-barbitone buffer (0.1M; pH8.6) was used with a current of 3.5ma (50v) for 2hr. The strips were stained with alcian blue. A, Fraction D1; B and C, alkali-treated fraction D1; D, bone sialoprotein.

faster-moving (peak B, Fig. 6b) having a mobility similar to fraction D3 and to pure chondroitin sulphate.

DISCUSSION

described previously. The elution patterns on DEAE-cellulose of fraction D1 before and after alkali treatment are shown in Fig. 6. Examination by cellulose acetate electrophoresis (Fig. 7) showed that fraction D1 was split by alkaline hydrolysis into two components, the slower-moving (peak A, Fig. 6b) having a mobility similar to, but slightly greater than, a sample of bone sialoprotein, and the

An estimate of the total amount of chondroitin sulphate in bone has been made by a DEAE-cellulose column method similar to that described in this paper (G. M. Herring & D. Oldroyd, unpublished work). Uronic acid determination after the elution of the chondroitin sulphate from papain digests indicated the presence of 780mg. of

chondroitin sulphate in 100g. of dry matrix. Similar experiments on EDTA extracts showed that at least 60% of the total uronic acid-containing material could be extracted by this procedure.

The chondroitin sulphate fraction isolated from EDTA extracts by the methods described contained about 11% of protein, which remained associated with the glycosaminoglycan throughout the subsequent separation procedures. From these results, it was concluded that the protein and chondroitin sulphate were most probably linked by covalent bonds. Further fractionation yielded two fractions, D1 and D2, which contained most of the protein, and a third fraction, D3, which contained relatively few amino acid residues.

The close similarities of the amino acid analyses of fractions D1 and D2 suggest that they may contain identical protein moieties, which, it may be assumed, also contain the glucosamine, hexose and sialic acid residues. The general composition and properties are indicative of a glycoprotein containing some 40% of carbohydrate, about half of which is sialic acid. This suggests possible similarities with the 'sialoprotein' which has been isolated previously from bone (Herring & Kent, 1963; Herring, 1964; Andrews & Herring, 1965) and which did not contain any detectable sulphate or uronic acid. A comparison of fraction D1, fraction D2 and 'sialoprotein', in terms of the constituents not derived from the chondroitin sulphate, has therefore been made (Table 4) and shows appreciable similarities. Some resemblances in amino acid composition were also noted (Table 3). If bone sialoprotein is identical with the glycoprotein moiety of fractions D1 and D2, then the situation is analogous to that found in the protein-polysaccharide complex of bovine nasal cartilage (Partridge, Whiting & Davis, 1965) where a uronic acid-free protein could be separated from that bound to chondroitin sulphate, both proteins having very similar amino acid compositions.

The differences in the ratio of protein to uronic

acid in the two fractions D1 and D2 may be attributable to differences in either the lengths or the number of chains of chondroitin sulphate attached to the glycoprotein 'core'. On the present results either or both of these variations are possible; heterogeneity in chain length would be reflected in the lack of resolution into clearly defined components on DEAE-cellulose, and the stepwise separation would represent a division of the range of molecular species into one group containing those with shorter or fewer chains (fraction D1) and a second group with longer or more chains (fraction D2). Hjertquist & Vejlsen (1966) found that chondroitin sulphate prepared by papain digestion of cortical bone from young dogs had a molecular weight range of 45 000–56 000, estimated from CPC solubility data. Though there are no comparable data available for bovine cortical bone, this relatively narrow molecular-weight range suggests that the number of chains, rather than chain length, may be the more important factor in the variation of chondroitin sulphate content in fractions D1 and D2.

The aldose composition of fraction D1 was obtained by paper chromatography and showed the presence of galactose, mannose, fucose and xylose. These sugars, with the exception of xylose, have been found in bone sialoprotein. The presence of this pentose and an alkali-labile bond between the chondroitin sulphate and glycoprotein moieties suggests that in fraction D1 the link between polysaccharide and peptide chains may be similar to that found in the chondroitin 4-sulphate-protein complex of bovine nasal septum, involving a galactosyl-xylosylserine grouping (Rodén & Smith, 1966).

Analytically, fraction D3 appeared to be relatively pure chondroitin sulphate; values for glucosamine and sialic acid were at the limits of detection, but there was still 0.9% of hexose (galactose) and 1.27% of protein present. Since the amino acid analysis of fraction D3 should be regarded as only semi-quantitative, conclusions cannot be reached concerning the possible relationship between the protein components of the three fractions, though there appeared to be some similarity of fraction D3 to fractions D1 and D2 (Table 3). However, the amount of protein is so small that it seems unlikely that this could represent an integral part of fraction D3. Even assuming a high molecular weight of 50 000 for the chondroitin sulphate chain, the most abundant amino acid, aspartic acid, would only amount to 0.83 of a residue/mole. Fraction D3 did not appear to resemble the protein-polysaccharide complex isolated by Muir & Jacobs (1967), which contained only about 2% of protein, or the chondroitin sulphate-peptides that result from the action of proteolytic digestion of the protein-polysaccharide complex (Muir, 1958; Anderson

Table 4. *Comparison of protein-bound chondroitin sulphate fractions D1 and D2 with bone sialoprotein*

These figures have been calculated from the compositions of fractions D1 and D2 (this paper) and bone sialoprotein (Andrews & Herring, 1965), and show the relative amounts of each constituent, expressed as a percentage of the sum of the constituents shown in the Table.

Fraction ...	Relative amount (% of sum of constituents shown)		
	D1	D2	Sialoprotein
Amino acids	59.7	63.9	56.8
Glucosamine	3.7	3.5	5.1
Hexose	11.3	12.3	13.4
Sialic acid	25.2	20.3	24.5

et al. 1965), since these possessed a relatively simple composition with three or four different amino acids, in particular serine, predominating. It is therefore possible that the peptide in fraction D3 represented contamination by a small amount of the protein-bound fractions. The apparent absence of xylose in fraction D3 might support this, though the presence of some other type of linkage cannot be excluded.

The fractions isolated in the present study do not resemble the protein-polysaccharide complexes isolated from epiphyseal (Castellani, 1965) and other cartilages. It has not been possible to detect any other uronic acid-containing components with the properties of these high-molecular-weight complexes in EDTA extracts. When collagenase was used to solubilize the entire matrix (Oldroyd & Herring, 1967), the uronic acid-containing components appeared to be closely similar to the fractions reported here in their behaviour on column chromatography and electrophoresis. However, 35% of the total chondroitin sulphate fraction (by papain digestion) was not recoverable from the collagenase digests. This might, at least in part, be explained by lower recoveries of protein-bound compared with protein-free chondroitin sulphate on DEAE-cellulose columns, but this does not exclude the possibility of other chondroitin sulphate-containing material which is not EDTA-extractable and not detectable in the separation procedure.

The present results are in accordance with those of Campo & Dziewiatkowski (1963) and Hirschman & Dziewiatkowski (1966) in a study of endochondral ossification by radioautographic and histochemical techniques. These authors suggest that, in the hypertrophic region of the epiphysis before calcification, degradation of the protein-polysaccharide complex occurs, possibly by proteolytic activity. This was confirmed by immunochemical studies from which the authors concluded that the protein had been extensively removed from the polysaccharide chains or else altered sufficiently to lose its immunochemical properties. The chondroitin sulphate fraction isolated from metaphyseal bone did not have the properties of a high-molecular-weight protein-polysaccharide complex similar to that isolated from epiphyseal cartilage under the same conditions. Since extensive resorption and remodelling is taking place in the metaphysis, both bone and calcified cartilage 'remnants' will be present, and the chondroitin sulphate obtained would be derived from both tissues.

It has been suggested that protein-polysaccharide complexes act as inhibitors of calcification (Glimcher, 1959) and that some type of degradation to a lower molecular weight is required to make the bound Ca^{2+} ions available or to remove diffusion barriers; a decrease in total chondroitin sulphate

itself does not appear to be required for initial calcification (Weatherell & Weidmann, 1963). Besides the results mentioned above, direct evidence for the presence of a proteolytic enzyme in ossifying cartilage (Jibril, 1967) and other histochemical studies (van den Hoof, 1964; Williamson & Vaughan, 1967) would be consistent with such a mechanism.

In adult diaphyseal bone, it can be assumed that the chondroitin sulphate results entirely from osteoblastic activity, and here calcification of the organic matrix (osteoid) proceeds rapidly as it is being laid down. The present results suggest that, in bone, the formation of a high-molecular-weight protein-polysaccharide complex may be restricted by the amount and type of protein 'core' available for attachment of polysaccharide chains. In bone 'sialoprotein' (mol. wt. 25 000; Williams & Peacocke, 1965) there are seven serine residues, some of which may be blocked by phosphate groups (Andrews & Herring, 1965). Though further evidence is required to show that the protein of fractions D1 and D2 is identical with bone sialoprotein, this would clearly provide such a limitation. A second process may be postulated in the case of the fraction D3 component, where the protein has apparently been largely removed from the polysaccharide chains either by the action of a peptidase or possibly by a specific glycosidase.

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