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COMPARATIVE STUDIES ON CARTILAGE AMINOPOLYSACCHARIDE SULPHATES

I. POLYSACCHARIDES FROM SHARK, SKATE, DOGFISH AND FIN WHALE

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SUMMARY

Studies on the polysaccharide constituents of cartilage from adult specimens of blue shark, skate and dogfish reveal the presence in each of these preparations of material corresponding to chondroitin sulphate and keratosulphate. However, each of the aminopolysaccharides is characterised by the presence of fractions of widely differing sulphate content. In contrast to the material from cartilaginous fish the polysaccharide mixture from fin-whale cartilage has chondroitin sulphate A as major constituent, both chondroitin sulphate C and keratosulphate being present in substantially lower amounts.

INTRODUCTION

It has now been established that mammalian cartilages are characterised by the presence of three types of aminopolysaccharide sulphate[1]. In general, chondroitin sulphate A (repeating unit: 3-*O*-β-D-glucuronido-*N*-acetyl-D-galactosamine 4-sulphate) and chondroitin sulphate C (repeating unit: 3-*O*-β-D-glucuronido-*N*-acetyl-D-galactosamine 6-sulphate) occur as main constituents, together with varying smaller amounts of keratosulphate (repeating unit: 4-*O*-β-D-galactosido-*N*-acetyl-D-glucosamine 6-sulphate).

Recent reports[2-8] reflect a substantial increase in interest in the aminopolysaccharide constituents of the connective tissues of cartilaginous fish. In this context LLOYD *et al.*[9] reported the isolation and infrared spectroscopic examination of polysaccharide sulphates from the cartilage of the blue shark (*Carcharhinus glaucus*), the dogfish (*Scyllium canicula*) and the skate (*Raja batis*). These observations have now been extended to include studies on the composition of these materials and of the sulphated aminopolysaccharides from the cartilage of the fin whale (*Balaenoptera physalus*).

MATERIALS AND METHODS

Aminopolysaccharide sulphate preparations

Preparations of aminopolysaccharide sulphate mixtures were obtained, as the potassium salts, from adult cartilage samples as described earlier.

Monosaccharide constituents of polysaccharide preparations

Portions (100–200 mg) of the polysaccharides dissolved in 10 ml of 1 N H_2SO_4 were heated in sealed tubes for 4–5 h at 110°. After cooling, the hydrolysates were treated with $Ba(OH)_2$ to pH 8.0 and then centrifuged to remove $BaSO_4$. Excess Ba^{2+} in the supernatants was then precipitated by gassing with CO_2 and the $BaCO_3$ precipitates removed by centrifuging. The products were then concentrated to dryness by rotary evaporation under reduced pressure at 35° and the residues redissolved in 0.5-ml portions of water.

The concentrated hydrolysates were chromatographed on Whatman No. 1 or 3MM papers using downward irrigation with phenol–ammonia–water[10] or ethyl acetate–pyridine–water (120:50:40, v/v) as solvents. The results of uni-dimensional separations were checked by two dimensional chromatography using combinations of phenol–water–ammonia[10], ethyl acetate–pyridine–water or butan-1-ol–acetic acid–water (50:12:25, v/v) as developing solvents.

Separated components were detected with aniline hydrogen phthalate[11] or *p*-anisidine[12] sprays for reducing substances and with Elson–Morgan reagents[13] for hexosamines. The nature of the hexosamine components was also ascertained by conversion to the corresponding pentose derivatives[14,15].

Chromatography on Ecteola-cellulose

Commercial grade Ecteola-cellulose (Brown Co., Berlin, N.Y.) was suspended in 0.5 M NaCl and, after packing in a glass column, treated successively with 0.5 M NaOH, 3.0 M NaCl and 0.1 M NaCl–0.1 M HCl (1:1, v/v), according to RINGERTZ AND REICHARD[16].

For analytical separations the modified cellulose was packed under gravity flow in a glass tube (15 cm × 1 cm) fitted at the lower end with a fritted glass disc. The column was then washed with 5 hold-up volumes of the 0.1 M NaCl–0.1 M HCl (1:1, v/v) mixture.

For the separation the polysaccharide (15–20 mg), dissolved in 2–4 ml of water, was converted to the free acid by passing through a Dowex-50 column (H^+ form, 50–100 mesh) and the acid eluate and washings concentrated to 1 ml by freeze-drying. The sample was then added to the column and allowed to drain into the resin bed under gravity flow. Elution of carbohydrate was achieved using a gradient produced by running 100 ml of 3.0 M NaCl–3.0 M HCl (1:1, v/v) into a mixing vessel fitted with a magnetic stirrer and containing 400 ml of 0.1 M NaCl–0.1 M HCl (1:1, v/v). The eluate from the column was collected in 5-ml fractions and the carbohydrate content of these determined with the phenol–sulphuric acid reaction[17].

On a preparative scale, the polysaccharides (3–4 g) were converted to the free acids as before, and then added to an Ecteola-cellulose column (60 cm × 3.5 cm). The column was then eluted in a stepwise manner using the appropriate concentrations of the NaCl–HCl (1:1, v/v) mixture indicated by the analytical experiments.

For the isolation of the polysaccharides from the eluates, fractions corresponding to homogeneous elution peaks were pooled and concentrated to 250 ml by rotary evaporation under reduced pressure at 37°. The solutions were then dialysed exhaustively against water for 24 h at 4°. The dialysate was then concentrated to 100 ml by rotary evaporation, passed through a Dowex-50 column (H^+ form) and the acid eluate and washings adjusted to pH 7.2 with aqueous KOH before freeze-drying.

Ethanol fractionation

For ethanol fractionation studies the aminopolysaccharide sulphates (1.5–2.0 g in 30 ml of water) were converted to the free acid form by passing through a Dowex-50 column (H^+ form, 50–100 mesh). The eluate and washings were diluted to 120 ml by the addition of water before adding 6.9 ml of glacial acetic acid and 6.3 g of calcium acetate. Ethanol was added to the mixture in a dropwise manner with stirring at room temperature to give a concentration of 37 % (v/v). Precipitation starting at this ethanol concentration was completed by the further addition of ethanol to 40 % (v/v) and keeping the mixture at 4° for 16 h. After separating by centrifuging, the precipitate was washed by suspension in 40 % (v/v) ethanol twice, with absolute ethanol and with ether using intermediate centrifuging, before drying *in vacuo* over $CaCl_2$.

To the 40 % (v/v) ethanol supernatant was added ethanol as before to give a final concentration of 50 % (v/v). After allowing the mixture to stand, precipitated material was collected and washed successively with 50 % (v/v) aqueous ethanol (twice), absolute ethanol and ether and dried as described above.

The calcium salts of the polysaccharide sulphates obtained by ethanol fractionation were converted to potassium salts after passing through a Dowex-50 column (H^+ form) and then precipitating in the presence of 80 % (v/v) ethanol, centrifuging and drying as already described.

Analytical methods

The ester sulphate contents of the preparations were determined gravimetrically[18] after hydrolysis in 3.5 N HCl at 100° for 20 h or spectrophotometrically[19] after sealed-tube hydrolysis in 1 N HCl at 110° for 5 h. Uronic acid was determined by the carbazole method[20] and hexosamine according to LEVY AND MCALLAN[21] after hydrolysis in 3.5 N HCl at 100° for 20 h. Nitrogen estimations were made with the micro-Kjeldahl apparatus[22] and potassium with the flame photometer. Analysis figures are corrected for loss in weight on drying the polysaccharides at 80° *in vacuo* over P_2O_5.

Infrared spectroscopy

Spectra were measured with the Perkin-Elmer Infracord spectrophotometer. All compounds were examined as mulls in Nujol.

EXPERIMENTAL AND RESULTS

The heterogeneity of crude aminopolysaccharide sulphate preparations

Analytical figures for the potassium salts of the crude aminopolysaccharide sulphate preparations from blue shark, dogfish, skate and fin whale are recorded in Table I. The analyses for each of these preparations are close to theoretical for "chondroitin sulphate-type" polymers except for the characteristic presence of "excess" sulphate in materials from blue shark, skate and dogfish.

However, paper chromatographic analysis of the acid hydrolysates of each of the crude preparations (Table II) suggested that each material was composed of more than one molecular species. The demonstration of D-glucuronic acid and D-galactosamine was in accord with the existence in each preparation of polysaccharide material similar to chondroitin sulphate, but the demonstration of D-galactose, D-glucosamine

TABLE I

ANALYTICAL FIGURES FOR CRUDE AMINOPOLYSACCHARIDE SULPHATE PREPARATIONS

Origin	*Nitrogen* (%)	*Hexosamine* (%)	*Uronic acid* (%)	*Ester sulphate* (%)	*Potassium* (%)
Blue shark	2.2	22.6	36.5	16.7	12.8
Skate	2.5	28.1	37.6	17.0	9.7
Dogfish	2.3	29.2	30.1	19.0	14.0
Fin whale	2.3	22.0	37.9	13.7	11.5

TABLE II

MONOSACCHARIDE CONSTITUENTS OF AMINOPOLYSACCHARIDE SULPHATE PREPARATIONS

Origin	*Glucuronic acid*	*Galactos amine*	*Glucos-amine*	*Galactose*	*Fucose*
Skate	++	++	++	++	+
Dogfish	++	++	++	++	+
Blue shark					
Unfractionated	++	++	++	++	+
0–40 % fraction	++	++	−	−	−
40–50 % fraction	−	−	++	++	+
Fin whale					
Unfractionated	++	++	+	+	Trace
0–40 % fraction	++	++	—	−	−
40–50 % fraction	+	+	+	+	Trace

and a trace of methylpentose suggested that the crude cartilage extracts also contain a molecular species corresponding to keratosulphate.

In addition, analytical scale fractionation of each of the polysaccharides on Ecteola-cellulose was marked by the elution of carbohydrate at widely differing molarities of the NaCl–HCl (1:1, v/v) mixture (see Fig. 1). The heterogeneity was most pronounced in the case of the blue shark preparation, but progressively less in the series skate, dogfish and fin whale.

Ethanol fractionation of aminopolysaccharide sulphate preparations from blue shark and fin whale

Fractionation of the aminopolysaccharide sulphate mixture from the blue shark between the limits of ethanol concentration of 0–40 % (v/v) and 40–50 % (v/v) yielded 1.15 g and 0.144 g of polysaccharide material respectively. When treated in an identical manner the fin whale preparation gave 1.1 g and 0.08 g of polysaccharide fractions respectively. Analysis figures for these preparations are given in Table III.

Examination of the monosaccharide constituents of the blue shark fractions revealed the separation of the original material into two polymer types. The fraction precipitating between 0–40 % (v/v) ethanol was found to contain only D-glucuronic acid and D-galactosamine while the 40–50 % fraction was characterised by its content of D-galactose and D-glucosamine with a trace of material corresponding in chromatographic mobility with L-fucose (see Table II). When examined similarly the 0–40 %

fraction from the fin whale polysaccharide mixture was found to contain only D-glucuronic acid and D-galactosamine but the 40–50% ethanol fraction contained D-galactose, D-glucosamine and L-fucose as well as trace amounts of D-glucuronic acid and D-galactosamine (Table II).

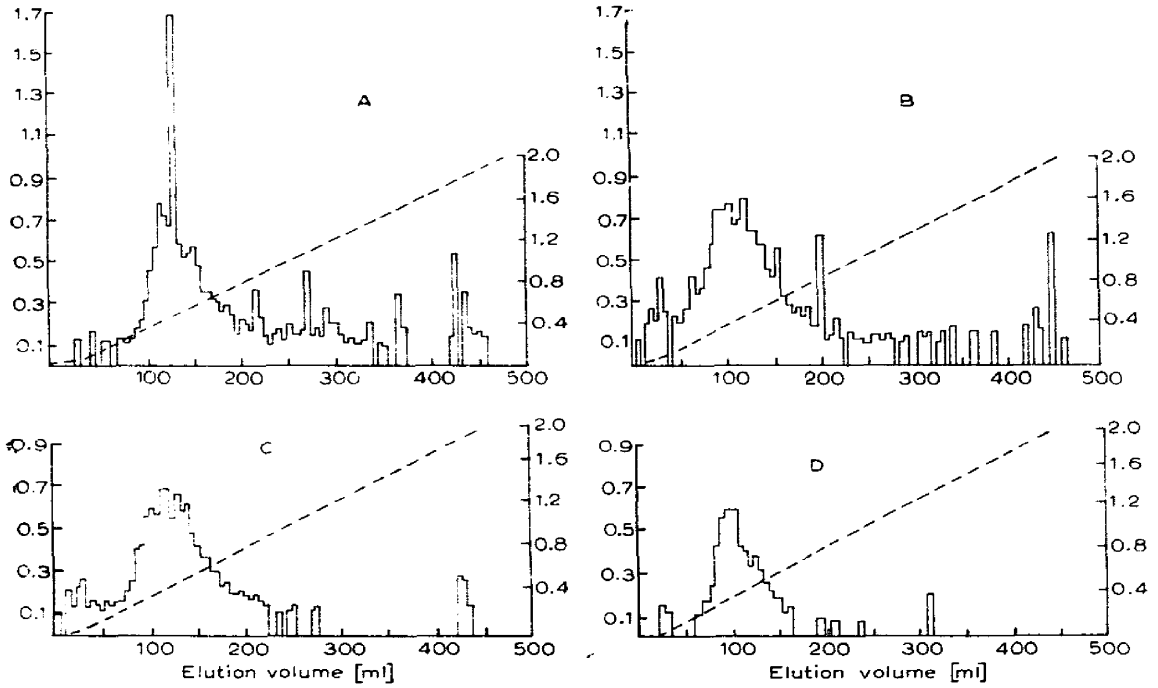

Fig. 1. Analytical scale fractionation of crude aminopolysaccharide sulphate preparations on Ecteola-cellulose. Right hand ordinate, extinctions of fractions at 485 mμ in the phenol–sulphuric acid reaction. Left hand ordinate, molarity of the NaCl–HCl (1:1, v/v) mixture. A, blue-shark preparation; B, skate preparation; C, dogfish preparation; D, fin-whale preparation.

Infrared spectra of aminopolysaccharide sulphate preparations

The results of the infrared spectroscopic examination of the crude aminopolysaccharide sulphates from blue shark, skate and dogfish and of the corresponding desulphated polymers in relation to that of mammalian chondroitin sulphates A and C

TABLE III

ANALYTICAL FIGURES FOR 0–40% AND 40–50% ETHANOL FRACTIONS FROM BLUE SHARK AND FIN WHALE

Origin	*Nitrogen* (%)	*Hexosamine* (%)	*Uronic acid* (%)	*Ester sulphate* (%)	*Potassium* (%)
Blue shark					
0–40%	2.4	22.1	32.2	16.7	12.6
40–50%	2.3	27.5	7.1	18.8	8.4
Fin whale					
0–40%	2.2	23.6	34.5	13.1	10.2
40–50%	2.3	26.4	31.2	12.9	10.9

have already been described[9]. The infrared spectrum of the crude aminopolysaccharide sulphate from fin-whale cartilage (Fig. 2A) exhibits the characteristic absorption at 1240 cm^{-1} and in the range 800–860 cm^{-1} attributable to vibrations involving the S–O and C–O–S linkages respectively. However, it is apparent that this spectrum bears a

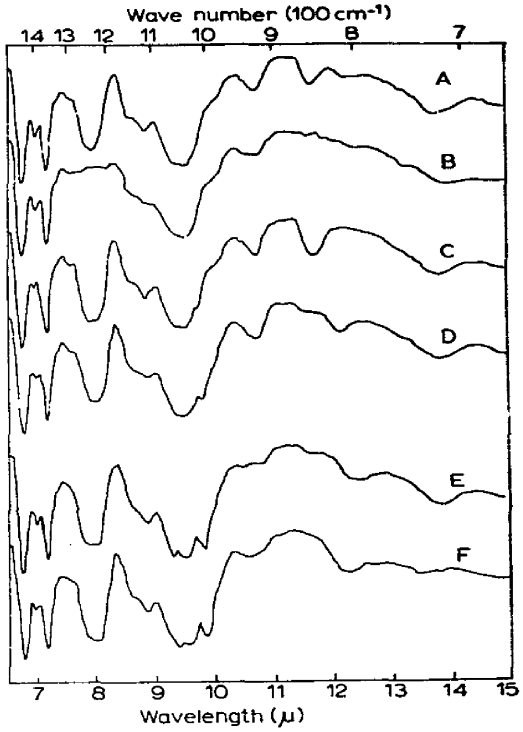

Fig. 2. Infrared spectra of polysaccharides from fin whale and blue shark. A, Crude fin-whale polysaccharides; B, chemically desulphated fin-whale polysaccharide; C, fin-whale 0–40% ethanol fraction; D, fin-whale 40–50% ethanol fraction; E, blue-shark 0–40% ethanol fraction; F, blue-shark 40–50% ethanol fraction.

closer resemblance to that given by material of bovine origin rather than the corresponding material from blue shark in that the 850 cm^{-1} band (axial C–O–S) is most pronounced while the 820 cm^{-1} band (equatorial C–O–S) is only of limited intensity. That absorption at 1240 cm^{-1}, 850 cm^{-1} and 820 cm^{-1} is associated with the ester sulphate group has been demonstrated by the disappearance of these bands from the infrared spectrum of a whale polysaccharide preparation desulphated chemically by the method of KANTOR AND SCHUBERT[23] (Fig. 2B).

The infrared spectrum of the 0–40% fraction from the whale polysaccharide (Fig. 2C) is marked by intensification of the band at 850 cm^{-1} and the absence of a band at 820 cm^{-1} while the spectrum of the 40–50% fraction has an intensified band at 820 cm^{-1} and only limited absorption at 850 cm^{-1} (Fig. 2D).

In contrast to the corresponding preparations from fin whale polysaccharide, the spectra of the blue shark 0–40% and 40–50% fractions both exhibit absorption attributable to the C–O–S vibration only at 820 cm^{-1} (Fig. 2E and 2F). However, the analytical scale fractionation of both of these materials on Ecteola-cellulose showed

that each preparation was still apparently composed of polymers of differing sulphate content (Fig. 3). It has been demonstrated previously that the degree to which polysaccharide sulphates are retained on Ecteola-cellulose is related directly to the ester sulphate content of the preparation[16]. To establish whether this was actually so in the present instance, 4 g of a 0–40 % (v/v) ethanol fraction from the blue shark was further fractionated on Ecteola-cellulose on a preparative scale. The elution pattern

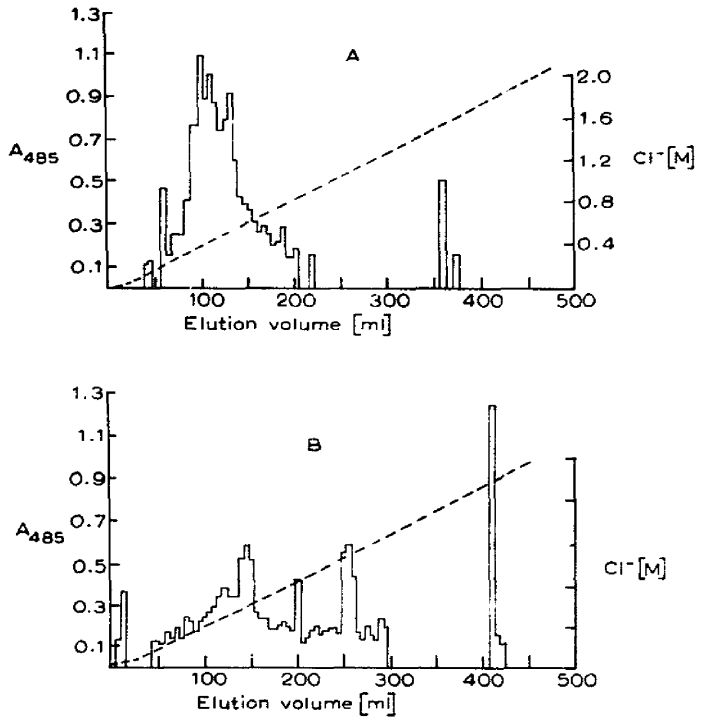

Fig. 3. Analytical scale fractionation of blue-shark 0–40 % fraction (histogram A) and 40–50 % fraction (histogram B) on Ecteola-cellulose. Ordinates as in Fig. 1.

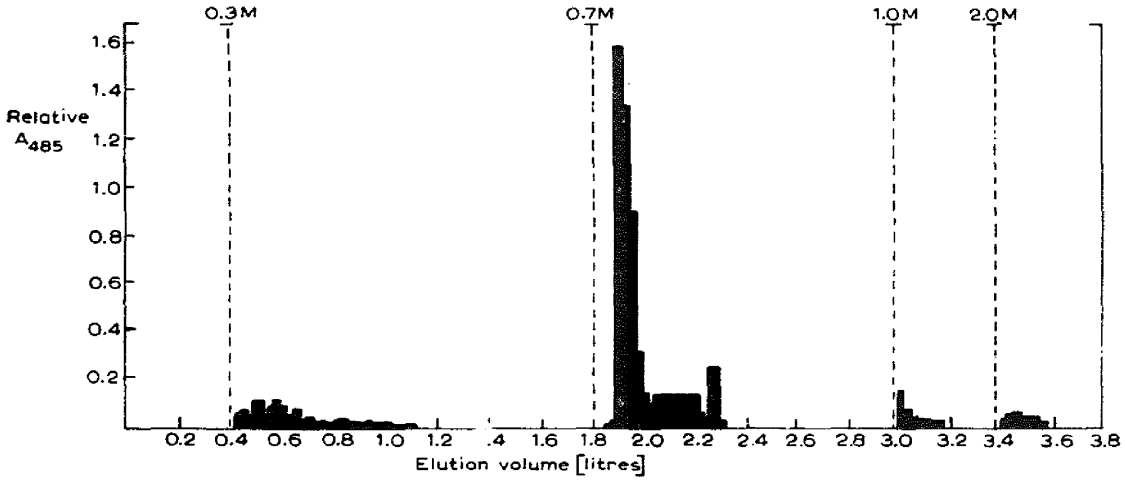

Fig. 4. Preparative scale fractionation of blue-shark 0–40 % ethanol fraction on Ecteola-cellulose.

obtained on treating the column in a stepwise manner with the NaCl–HCl (1:1, v/v) mixture at concentrations of 0.3 M, 0.7 M, 1.0 M and 2.0 M is shown in Fig. 4. The analytical figures for the potassium salts of the polysaccharides isolated from eluates corresponding to these molarities (Table IV) reflect the increasing sulphate contents of the molecular species in the preparation.

TABLE IV

ANALYTICAL FIGURES FOR POLYSACCHARIDE SULPHATES ISOLATED AFTER THE ECTEOLA-FRACTIONATION OF THE BLUE-SHARK 0–40% ETHANOL FRACTION

Eluant (M)	*Yield (g)*	*Nitrogen (%)*	*Hexosamine (%)*	*Uronic acid (%)*	*Ester sulphate (%)*	*Potassium (%)*
0.3	0.72	2.6	20.4	34.5	14.1	11.6
0.7	1.84	2.25	23.0	39.5	17.4	14.0
1.0	0.105	2.5	—	—	18.3	—
2.0	0.022	—	—	—	22.6	—

DISCUSSION

It is apparent from the present work that the aminopolysaccharide sulphate esters constituting the matrix of cartilage from blue shark, dogfish and skate differ substantially from corresponding polymers present in the cartilage of both terrestrial and aquatic mammals. Polymer species similar to mammalian chondroitin sulphate and keratosulphate have been demonstrated but these are characterised by their widely differing contents of ester sulphate. Although the existence in elasmobranch cartilage of highly sulphated polysaccharides has formed the subject of earlier reports[2–8], little is known regarding the effect of ester sulphate content on the properties of these materials. The methods presently described permit the isolation of polysaccharide species of defined sulphate content suitable for further structural and enzymological study.

The sulphated aminopolysaccharides from fin-whale cartilage closely resemble preparations from terrestrial mammals in that although containing chondroitin sulphate and keratosulphate, fractionation of the preparation on Ecteola-cellulose reveals that there is only limited heterogeneity as regards ester sulphate contents of the constituents (*cf.* RINGERTZ AND REICHARD[16]). In particular, the presence in the infrared spectrum of strong absorption at 850 cm^{-1} both for crude preparations and the 0–40% fraction suggests that material identical in structure to chondroitin sulphate A is the principle polysaccharide constituent of the original matrix, condroitin sulphate C and keratosulphate (both of which give rise to absorption at 820 cm^{-1}) being present in quantitatively lesser amounts.

The consistent demonstration of a methylpentose similar to L-fucose as a monosaccharide constituent in all of the crude preparations, and its specific association with D-galactose and D-glucosamine in the 40–50% fraction from blue shark, deserves special mention. Mammalian keratosulphate preparations, having D-galactose and D-glucosamine as principle monosaccharide constituents, have been reported consistently as containing traces (1–2%) of a methylpentose component[24]. In contrast, vertebral cartilage induced to form in tissue cartilage and not containing kerato-

sulphate (as shown by the absence of D-galactose and D-glucosamine) was also free from methylpentose[25]. Although these observations present evidence for the firm association of methylpentose components with keratosulphate fractions, it is not known how these moieties are involved structurally in the polymer molecule[26].

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