The Structure of Keratan Sulphates from Various Sources

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Quantitative structural comparisons were made between keratan sulphates isolated from various sources, namely pig nucleus pulposus, bovine cornea, and the costal cartilages of children, a young adult with Marfan syndrome and of old human autopsies. In human costal cartilage the amount of keratan sulphate increases markedly with age, although total mucopolysaccharide decreases to some extent, concomitant with a decrease in chondroitin 4-sulphate and an increase in chondroitin 6-sulphate. Comparison of molecular weights estimated by gel chromatography with those calculated from the molar ratio of galactose to mannose indicates that keratan sulphates of human costal cartilages of children and of a young adult with Marfan syndrome, and of pig nucleus pulposus, contain one mannose residue per chain, whereas keratan sulphates of old human costal cartilage and of bovine cornea contain one to two, and two, per chain respectively. After mild acid-catalysed desulphation of pig nucleus pulposus keratan sulphate, approx. 12% of the mucopolysaccharide aggregates irreversibly once the water is removed from the polysaccharide. The following conclusions have been drawn from a methylation analysis of keratan sulphates of various sources, aided by g.l.c.-mass spectrometry. (1) Fucose and *N*-acetylneuraminic acid are non-reducing terminal residues and the sialic acid is linked to the 3-position of galactose residues. (2) Pig nucleus pulposus keratan sulphate has approximately four non-reducing terminal groups per molecule and appears to be slightly less branched than the costal-cartilage keratan sulphate of children. The branching in human costal-cartilage keratan sulphates decreases with age. Bovine corneal keratan sulphate appears to be unbranched. (3) Mannose residues are linked by three different substituents in human costal-cartilage and bovine corneal keratan sulphates, and by two different substituents in pig nucleus pulposus keratan sulphate. (4) The sulphate ester groups are all on the 6-position of *N*-acetylglucosamine and galactose residues. The degree of sulphation increases with age in costal keratan sulphates with the increase mainly of the galactose 6-sulphate residues.

Keratan sulphates isolated from cornea (Meyer *et al.*, 1953), nucleus pulposus (Antonopoulos *et al.*, 1961; Lohmander *et al.*, 1973) and cartilage of various vertebrates (Meyer *et al.*, 1958; Kaplan & Meyer, 1959), exhibit a high degree of heterogeneity (Seno *et al.*, 1965; Mathew & Cifonelli, 1965) and polydispersity (Laurent & Anseth, 1961). The heterogeneity resides in their sulphate, sialic acid and L-fucose contents, in their distribution of ester sulphate groups between the hexosaminyl and galactosyl moieties of the repeating disaccharide units (Bhavanandan & Meyer, 1966), in their linkage between carbohydrate and peptide (Seno *et al.*, 1965), and in the extent of branching.

The keratan sulphates of bovine cornea and old human rib cartilage were shown by methylation studies to be polymers consisting of the repeating unit (1→3)- β -D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy- β -D-glucopyranose sulphated in the 6-position of glucosaminyl (Hirano *et al.*, 1961)

and galactosyl (Bhavanandan & Meyer, 1966, 1967, 1968) moieties.

The structure of keratan sulphate of pig nucleus pulposus as a polymer consisting mainly of the repeating disaccharide unit (1→3)- β -D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy- β -D-glucopyranose sulphated on the 6-positions of both the glucosaminyl and galactosyl moieties has now been established with the use of the Hakomori (1964) methylation technique, which gives better yields and less depolymerization with some polysaccharides (Cheetham & McIlroy, 1969). Quantitative structural comparisons are made between keratan sulphates isolated from various sources; pig nucleus pulposus, bovine cornea, and the costal cartilages of children, of a young adult with Marfan syndrome and of old human autopsies. Some of these results were presented at the Second International Santa Catalina Colloquium on Extracellular Matrix Influences of Gene Expression.

Experimental

Materials

Human costal cartilages were collected from 60- to 91-year-old autopsies, from a 23-year-old adult with Marfan syndrome, and from 4-9-year-old children. Nucleus pulposus was collected from approx. 2-year-old pigs. Cornea was obtained from 2-3-year-old cows.

The commercial enzymes employed were bovine testicular hyaluronidase (Type VI, H-2376; Sigma Chemical Co., St. Louis, Mo., U.S.A.), papain (NO96TJ; Winthrop Corp., New York, N.Y., U.S.A.) Pronase (B Grade, 101185; Calbiochem, Los Angeles, Calif., U.S.A.), neuraminidase (Sigma, type V, N-2876) and β -exo-D-galactosidase (code BG; Worthington Biochemical Corp., Freehold 2, N.J., U.S.A.)

Preparation of keratan sulphate fractions

Keratan sulphate fractions from human costal cartilages and bovine cornea were prepared by the procedures described previously (Seno *et al.*, 1965). Pig nucleus pulposus keratan sulphate was prepared by the following procedure. Acetone-dried nucleus pulposus (55g) ground in Wiley mill was suspended in 450ml of 0.06M-sodium citrate buffer, pH 5.5, containing 0.03M-EDTA, 0.03M-cysteine and papain (2g) activated by heating the latter for 1h at 60°C. The solution was incubated for 2 days at 65°C with one addition of the enzyme (1g) after the first day. The insoluble material was removed by centrifugation and the solution brought to 0.16 M-calcium acetate and 0.25M-acetic acid. The mucopolysaccharides were precipitated with 2 volumes of ethanol. The mixture was chilled overnight and centrifuged. The precipitate was washed with 80% ethanol, dissolved in 300ml of 1M-sodium acetate (pH 5) and the solution was stirred with chloroform-*n*-amyl alcohol. After removal of the organic solvent, the mucopolysaccharides were recovered by precipitation with 2 volumes of ethanol, washed with 80% ethanol, dissolved in 0.01M-calcium acetate buffer (pH 7.6) and incubated with Pronase (2.5g) at 37°C under toluene. Pronase (1g) was added at 24h intervals while the pH was maintained at 7.2-7.8 with 1M-NaOH. The digestion was continued until no further change in the pH was observed. At the end of the digestion, the pH was adjusted to 4.5 with acetic acid and the digest was treated with a mixture of Lloyd's (5895; Harleco, Philadelphia, Pa., U.S.A.) reagent-kaolin (3:1) followed by centrifugation. The residue was washed with 1M-sodium acetate. The mucopolysaccharides were precipitated from the supernatant with 2 volumes of ethanol, centrifuged, washed with 80% ethanol and dissolved in 300ml of water. The solution was brought to 5% with respect to calcium acetate, chilled overnight and centrifuged. The precipitate was washed with 5% calcium acetate and discarded. The combined supernatant

was brought to 0.5M with respect to acetic acid and fractionated with 32, 45, 55 and 65% ethanol.

The first two ethanol fractions consisted mostly of chondroitin sulphate with a small amount of hyaluronic acid. The 55%-ethanol fraction containing 68% keratan sulphate and 32% chondroitin sulphate, and the 65%-ethanol fraction consisting of 90% keratan sulphate and 10% chondroitin sulphate, were further fractionated through a Bio-Gel P-100 column (2.5cm $\times$ 200cm) with 0.5M-NaCl as eluent. The effluent was assayed for hexose and uronic acid. The keratan sulphate peak partially overlapped, and was slightly ahead of, that of chondroitin sulphate. By pooling the portion of the keratan sulphate in the peak free of chondroitin sulphate, approx. 65% of total keratan sulphate in a highly pure form was obtained by precipitation with 3 volumes of ethanol. Some of the chemical analysis of the preparation is given in Table 1. This fraction was used in most of the present studies. The rest of the fraction whose keratan sulphate could not be separated from chondroitin sulphate by the gel filtration was digested with testicular hyaluronidase in 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-NaCl for 2 days at 56°C with one addition of the enzyme after 24h of incubation. After treatment with the mixture of Lloyd's reagent-kaolin as described above, the keratan sulphate was purified by gel filtration through the Bio-Gel P-100 column, eluting with 0.5M-NaCl.

Analytical procedures

Analytical procedures, described in part previously (Choi & Meyer, 1975), were the following: Hexose (anthrone; Scott & Melvin, 1953); uronic acid (carbazole; Dische, 1947); ester sulphate [benzidine (Antonopoulos, 1962) or rhodizonate (Terho & Hartiala, 1971)]; sialic acid [resorcinol (Svennerholm, 1957) or thiobarbituric acid (Warren, 1959)], reducing sugar (ferricyanide; Park & Johnson, 1949); fucose [cysteine-sulphuric acid (Dische & Shettles, 1948) or g.l.c.]. Neutral sugars were also determined by a borate column with a Technicon automatic sugar analyser, and hexosamine by a Beckman amino acid analyser (Choi & Meyer, 1974). Quantitative analysis of chondroitin sulphate isomers was achieved with specific chondroitinases and sulphatases (Yamagata *et al.*, 1968).

G.l.c. analysis of neutral sugars and hexosamines, as their alditol acetates, were carried out with a 6ft (1.83m) glass column packed with 3% (w/w) ECNSS-M on Gas Chrom Q (Sawardeker *et al.*, 1965; Kim *et al.*, 1967) under the conditions described previously (Choi & Meyer, 1974). Combined g.l.c. and mass spectrometry was carried out on a Varian Aerograph 1400 gas chromatograph coupled to a Du Pont model 21-491 mass spectrometer. Partially methyalted neutral sugars and *N*-methyl-hexosamines in the hydrolysates of permethylated

keratan sulphate were analysed as their alditol acetates by g.l.c. and identified by their retention times and mass spectra as described previously (Choi & Meyer, 1974).

Exo-D-galactosidase digestion of pig nucleus pulposus

Exo-D-galactosidase was obtained by CM-cellulose column chromatography of a cell-free extract of *Coccobacillus* (Choi & Meyer, 1975). It contained both α - and β -exo-D-galactosidases (tested with α - and β -D-nitrophenyl-D-galactopyranosides as substrates), had a weak hydrolytic activity for β -D-nitrophenylglucosaminide, but was negative for endo-galactopyranose activity. The pig nucleus pulposus keratan sulphate (0.5 mg), after removal of sialic acid by neuraminidase, was incubated with the enzyme preparation (0.3 mg of protein) in pH 7.0 buffer (0.1 M- Na_2HPO_4 containing KH_2PO_4) for 3 days at 37°C and the digest was analysed on a Technicon automatic sugar analyser. In another experiment keratan sulphate was incubated with the β -exo-D-galactosidase from *Escherichia coli* under the same conditions.

Amidation and N-acetylation

Amidation of carboxyl groups and N-acetylation of amino groups of terminal amino acids of the peptides were carried out essentially as described previously (Stern *et al.*, 1971). Each keratan sulphate preparation (50 mg) was converted to the pyridinium salt by passage through a column (1 cm $\times$ 50 cm) of AG50-X8 resin (H^+ form) collecting the effluent in the presence of 5 drops of redistilled pyridine. The column was washed with water (40 ml) and the combined solution was freeze-dried. The dried material was suspended in redistilled dimethyl-formamide (10 ml) containing 7 drops of pyridine. The suspension was shaken for 1 h at 50°C, then thionyl chloride (0.12 ml) was added and the reaction mixture was shaken for another 4 h at room temperature, followed by addition of diethylamine (2 ml). The solution was left overnight at room temperature and the polysaccharide was isolated by the addition of diethyl ether (60 ml) followed by centrifugation. It was then re-precipitated from aqueous solution (15 ml) by three volumes of ethanol, centrifuged, washed with 80% ethanol, and N-acetylated by the following procedure: The residue (20 mg) dissolved in water (2 ml) was mixed with acetic anhydride (0.2 ml) and 5% (w/v) sodium bicarbonate (0.8 ml). After 1 h at room temperature the solution was adjusted to approx. pH 7 with 5% sodium bicarbonate. The additions of acetic anhydride (0.2 ml) and 5% sodium bicarbonate (0.8 ml) and the pH adjustment after the reaction for 1 h at room temperature were repeated three times. The N-acetylated product was recovered by alcohol precipitation and washed three times with 80% ethanol.

Estimation of carbohydrate chain length of keratan sulphate by gel filtration after treatment with alkali-borohydride

A calibrated column (1.28 $\times$ 116 cm) of Bio-Gel P-100 (100–200 mesh) was used. The gel was prepared according to the Bio-Rad literature. The qualities of the packing, the void volume and the salt region were ascertained with Blue Dextran 200 and Na_2SO_4 . The column was stabilized for 24 h with 0.5 M-NaCl solution and the elution continued until the column was used. The calibration was carried out with a 2 h digest of chondroitin sulphate C isolated from pig nucleus pulposus with testicular hyaluronidase in 0.1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl at 56°C. The effluents were analysed by the carbazole reaction, the Elson–Morgan reaction for total hexosamine, reducing-sugar value (Park & Johnson, 1949) and the Morgan–Elson reaction (Reissig *et al.*, 1955) with N-acetylchondrosine as standard. Molecular weights of each fraction were then calculated for the calibration of the column as described previously (Choi & Meyer, 1974). In order to estimate and compare the carbohydrate chain length, N-acetylated and amidated keratan sulphates of different sources were treated under N_2 in (a) 0.5 M-NaOH containing 0.5 M- NaBH_4 for 24 h at 24°C and (b) 0.05 M-NaOH containing 1.0 M- NaBH_4 for 48 h at 37°C. After removal of NaBH_4 as methyl borate, products were analysed for hexosamine, hexosaminitol (Choi & Meyer, 1974) and amino acids by the amino acid analyser, and chromatographed on the calibrated Bio-Gel P-100 column with 0.5 M-NaCl as eluent, the flow rate being maintained throughout the experiments. The elution pattern was monitored by the anthrone reaction and the molecular weight of the elimination product in each peak was estimated from the calibration data described previously (Choi & Meyer, 1974).

Desulphation

Two methods of desulphation were compared. For mild acid-catalysed desulphation (Hirano *et al.*, 1961), dried keratan sulphate of pig nucleus pulposus (20 mg) was shaken with anhydrous methanol (2 ml) in a tube with Teflon-lined screw cap for 3 h at 24°C. Dry AG 50-X8 resin (H^+ form, 200 mg) and dry NaCl (40 mg) were added, and the heterogeneous mixture was shaken for 48 h at the same temperature. After the addition of water (8 ml) and shaking for another 18 h at 4°C, the reaction mixture was neutralized with 0.5 M-NaOH, filtered through a pad of glass wool, and the resin on the pad was washed twice with water.

Base-catalysed desulphation was carried out essentially as described previously (Choi & Meyer, 1975). Dried keratan sulphate (10 mg) was converted to its pyridinium salt by passing it through a small

column (5ml) of AG50-X8 resin (H^+ form), eluting with water (20ml) and collecting the eluate in a container with three drops of pyridine in an ice bath. The effluent was evaporated *in vacuo* and dried thoroughly. The residue was dissolved in dimethyl sulphoxide containing 2% (v/v) pyridine at 60°C for 10min and heated at 100°C for 9h. The organic solvent was removed by distillation *in vacuo*, and inorganic sulphate by the addition of an equivalent amount of barium acetate followed by centrifugation, or by an anion-exchange column.

Gel filtration of desulphated keratan sulphate

In order to study the extent of depolymerization, the desulphated product was chromatographed on a pre-calibrated Bio-Gel P-100 column (1 cm $\times$ 100 cm). The column was packed after swelling the gel in formamide for 24h at room temperature. The column was stabilized by eluting with formamide solution for 2 days. Formamide was used as eluent and the flow rate was maintained throughout the experiments and the distribution coefficients (K_d) were calculated (Gelotte, 1960).

Methylation of keratan sulphate

For methylation the Hakomori procedure (Hakomori, 1964; Choi & Carubelli, 1968) followed by a modified Purdie technique (Purdie & Irvine, 1903) as described previously (Choi & Meyer, 1975) was used. A stream of N_2 gas was supplied constantly throughout. A stock solution of methylsulphonyl-carbanion (0.5 mequiv./ml) was prepared (Choi & Meyer, 1975) and stored under N_2 at -27°C until used. In a typical experiment, pig nucleus pulposus keratan sulphate (20mg) was dissolved in redistilled dimethyl sulphoxide (5ml) by shaking at 60°C for 30min and cooled. The solution was mixed with the carbanion (10ml, 0.5 mequiv.) and stirred gently for 6h at room temperature. Redistilled methyl iodide (2ml) was added dropwise, the reaction mixture was kept at room temperature, and was stirred overnight. The additions of the carbanion solution (10ml) and methyl iodide (0.5ml) were repeated twice at the same intervals. The reaction mixture was freeze-dried after the addition of water (100ml). The product, after being dissolved in water (30ml), was extracted twice with chloroform (60ml) and the chloroform layer was washed with water. The product in the aqueous layer, after concentration to a small volume, was chromatographed on a Bio-Gel P-2 column (1.50 cm $\times$ 118 cm), pre-washed with dimethyl sulphoxide (30%) and dimethylformamide (30%) and eluted with 10% ethanol. The methylated product (monitored by the anthrone reaction) eluted at the void volume was freeze-dried (yield 17.0mg). The product in the anthrone-positive chloroform layer was isolated by gel filtration on a Sephadex LH-20 column (1.50 cm $\times$ 106 cm) and was eluted with

chloroform. The anthrone-positive product was pooled and freeze-dried (yield 1.6mg). Both products were combined and subjected to Purdie's methylation procedure by dissolving in dimethylformamide (1.5ml) in a tube with a Teflon-lined screw cap. Freshly prepared silver oxide (500mg) and methyl iodide (1ml) were added to the tube which was covered with aluminium foil and shaken for 24h at room temperature. After the addition of water (10ml) the solution was extracted twice with chloroform (20ml) and the methylated products in the aqueous and chloroform layers were isolated by gel filtration as described before. The combined product (white cotton-like) after freeze-drying yielded 16.2mg.

Methylation of desulphated keratan sulphate

The procedure was essentially similar to that described above. Desulphated pig nucleus pulposus keratan sulphate (10mg) was dissolved in redistilled dimethyl sulphoxide (2ml) followed by three additions of the carbanion (2ml) and methyl iodide (0.5ml) at the same intervals. The reaction mixture was then passed through the Sephadex LH-20 column eluted with chloroform and the eluate monitored by the anthrone reaction. After pooling and removing the solvent, the product was subjected to two further treatments with Purdie's reagents as described above. The reaction product was isolated by gel filtration on the same column, with chloroform as eluent (yield 8.4mg).

Hydrolysis of the permethylated products for g.l.c. and g.l.c.-mass-spectrometric analyses

The methylated product was hydrolysed either by formolysis followed by acid hydrolysis (Yang & Hakomori, 1971; Choi & Meyer, 1975) or by acetoysis followed by acid hydrolysis (Stellner *et al.*, 1973). The latter method gave a better yield. The permethylated keratan sulphate (0.5mg) in a tube with Teflon-lined screw cap was dissolved in 0.5M- H_2SO_4 in 95% acetic acid (0.5ml), and heated for 16h at 80°C. After the addition of water (0.5ml), the reaction mixture was heated for an additional 5h at 80°C and passed through a column (0.5 cm $\times$ 5 cm) of AG3-X 4A resin (acetate form), which was washed with methanol (10ml). After the removal of methanol, the residue was dissolved in 0.3ml of aq. $NaBH_4$ (10mg) and left overnight at room temperature. Excess borohydride was destroyed by acetic acid (3 drops) and removed as methyl borate by repeated evaporation with anhydrous methanol, and dried *in vacuo*. The residue was acetylated with redistilled acetic anhydride (0.5ml) in a tube with a Teflon-lined screw cap for 3h at 120°C. Acetic anhydride was removed by co-evaporation with toluene in a rotary evaporator. The residue was extracted with methylene chloride, evaporated to

0.1 ml and portions (2–3 μ l) were injected in g.l.c. or g.l.c.–mass spectrometry equipment for analysis. For routine quantitative analysis the column temperature was programmed from 170° to 200°C, and for g.l.c. retention-time and mass-spectral analyses, the column was kept isothermal at 170°C for the partially methylated neutral sugar alditol acetates, and at 190°C for the partially methylated *N*-methylhexosaminitol acetates.

Oxidation of partially methylated sugar alditols from permethylated keratan sulphate

The partially methylated sugar alditols (1 mg), prepared from the permethylated keratan sulphate as described above, were oxidized in aq. 0.25 M-sodium metaperiodate (0.5 ml) at room temperature for 18 h. The excess periodate was destroyed and the products reduced with NaBH₄ (20 mg) for 3 h at room temperature. The excess borohydride was destroyed by the addition of acetic acid (10 drops) and removed as methyl borate, as before. The iodate in the reaction mixture was removed by passage through a column (5 ml) of AG1-X8 resin (acetate

form); the eluent was water (30 ml). The product in the effluent was recovered by evaporation and dried thoroughly before acetylation for g.l.c. analysis as described above.

Results and Discussion

Keratan sulphates were isolated, after extensive proteolysis with crude papain and Pronase, from human costal cartilages of 4–9-year-old children, of a 23-year-old adult with Marfan syndrome, and of over-60-year-old autopsies, from 1–2-year-old pig nucleus pulposus and 2–3-year-old bovine cornea, and fractionated by alcohol precipitation. The fractions were further purified by gel filtration before and after digestion with hyaluronidase. The isolated fractions contained not more than 5% of amino acids. Xylose could not be detected by g.l.c. in any of the isolated products, indicating the absence of chondroitin sulphate. Some of the molar ratios are summarized in Table 1.

As previously reported by us (Kaplan & Meyer,

Table 1. *Analysis of native keratan sulphates from different sources*

Neutral sugars were analysed by g.l.c. as alditol acetates, and by a Technicon sugar analyser as borate complexes. Hexosamines were determined by g.l.c. as alditol acetates and by a Beckman amino acid analyser.

Compounds	Source of keratan sulphate ...	Molar ratio				
		Costal cartilage			Pig nucleus pulposus	Bovine cornea
		Children	Young adult with Marfan syndrome	Old human		
Galactose/Glucosamine		1.26	1.22	1.21	1.29	1.05
Sulphate/Glucosamine		1.16	1.35	1.45	1.50	1.26
Fucose/Mannose		3.37	2.88	1.93	0.53	0.35
Sialic acid/Mannose		3.88	2.13	1.35	3.88	0.41

Table 2. *Mucopolysaccharide content in different sources*

Source of keratan sulphate ...	Mucopolysaccharide content (%)			
	Costal cartilage			Pig nucleus pulposus
	Children	Young adult with Marfan syndrome	Old human	
Mucopolysaccharide in acetone-dried powder*	14.0	17.7	12.0	46.2
Of total mucopolysaccharide				
keratan sulphate	24.9 (3.3)†	32.0 (5.7)†	61.9 (7.9)†	21.0 (9.3)†
chondroitin sulphate	75.1	68.0	38.1	79.0
Of total chondroitin sulphate				
chondroitin 4-sulphate‡	50.3	43.2	36.3	19.0
chondroitin 6-sulphate‡	49.7	56.8	63.7	81.0

* Each tissue had small amounts of hyaluronic acids; for example, 0.63% of total mucopolysaccharide was hyaluronic acid in pig nucleus pulposus.

† Values in parentheses are % by weight of acetone-dried powder.

‡ Analysed with specific chondroitinases and sulphatase (Yamagata *et al.*, 1968).

1959) and by others (Mathew & Cifonelli, 1965), the total mucopolysaccharide content in cartilaginous tissues and the relative amounts of chondroitin 4- and 6-sulphates and keratan sulphate vary with age and sources (Table 2). In human costal cartilage the keratan sulphate increases markedly with age and the total mucopolysaccharide decreases to some extent, concomitant with a decrease in chondroitin 4-sulphate and an increase in chondroitin 6-sulphate. Although only approx. one-fifth of the mucopolysaccharide is keratan sulphate, the pig nucleus pulposus yields the highest amount of the keratan sulphate among the tissues studied (Table 2).

Comparison of chain length of keratan sulphate from different sources

Molecular weights of keratan sulphates were estimated by gel filtration after treating the keratan sulphates with alkali, and the results were compared to the calculated values based on the molar ratio of galactose to mannose. In order to obtain the maximum alkaline elimination of the carbohydrate chains from the peptide backbone of the keratan sulphates, the amino and carboxyl ends of the peptide were coupled with acetyl and diethylamino groups respectively. Of the two conditions studied (a) 0.5M-NaOH in the presence of 0.5M-NaBH₄ for 24 h at 24°C and (b) 0.05M-NaOH in the presence of 1.0M-NaBH₄ for 48 h at 37°C, the condition (b) resulted in more extensive overall destruction of threonine and serine, concomitant with a higher loss of galactosamine. Also noteworthy was that the loss of the threonine was invariably higher under condition (a), whereas the condition (b) effected a higher loss in serine residues (H. U. Choi & K. Meyer unpublished results). The gel filtration through a Bio-Gel P-100 column of the elimination products obtained under the two conditions showed that the product obtained under condition (b) produced an elution profile with a higher *K_d* than that under condition (a), which indicated a greater release of the carbohydrate chains from the peptide core under the latter condition. As previously reported (Choi

& Meyer, 1974), a study with the sulphated glycoprotein isolated from chick allantoic fluid demonstrated that almost complete release of the carbohydrate chains from the protein backbone was achieved under condition (b).

Masking only the amino group with acetyl and both the amino and carboxyl groups of terminal amino acids of the peptide backbone with acetyl and diethylamino groups respectively, followed by alkali-borohydride treatment under condition (b) resulted in more extensive elimination of the carbohydrate chains, as demonstrated by the increased destruction of threonine and serine, concomitant with a higher loss of galactosamine (Table 3). The result suggests that at least some of the serine or threonine residues, which are linked to the carbohydrate chains, are at terminal positions of the attached peptide.

The molecular weights of the elimination products of skeletal keratan sulphate (keratan sulphate II) were estimated by gel filtration on a calibrated Bio-Gel P-100 column as described in the Experimental section and comparison was made with those calculated from the molar ratios of galactose to mannose (by multiplying by the molecular weight of the sodium salt of the repeating disaccharide monosulphate). The bovine cornea keratan sulphate (keratan sulphate I), which was digested exhaustively with papain and Pronase, is included in the comparison, and the results are summarized in Table 4. The molecular weights estimated by gel filtration range from approx. 10000 to 12000 whereas the calculated values range from 5400 to 13000. The results indicate that keratan sulphate II of pig nucleus pulposus and human costal cartilages of children and a young adult with Marfan syndrome contained one mannose residue per polysaccharide chain. There are, however, discrepancies in keratan sulphate II of old human costal cartilage and keratan sulphate I between the values obtained by the two different methods. It could be that old human costal keratan sulphate II is composed of a mixture of polysaccharide chains which contain one to two mannose residues

Table 3. Loss of threonine, serine and galactosamine by alkali-borohydride treatment of keratan sulphate before and after masking terminal amino acids

Each keratan sulphate preparation was treated with 0.05M-NaOH in the presence of 1.0M-NaBH₄ for 48 h at 37°C.

			Loss (% μ mol)								
Keratan sulphate treatment			Native			N-acetylated			N-acetylated and amidated		
Source	Compound lost	...	Thr	Ser	GalN	Thr	Ser	GalN	Thr	Ser	GalN
Costal cartilage of											
Children			70.5	78.9	79.0	78.0	83.7	85.2	75.4	89.1	89.5
Young adult with Marfan syndrome			68.2	64.0	76.3	77.0	75.7	84.2	76.8	85.2	88.7
Old human			66.7	48.4	73.4	81.5	60.4	82.5	74.8	77.9	87.3
Pig nucleus pulposus			39.9	36.8	72.2	76.2	52.6	80.5	79.7	68.0	87.6

Table 4. Comparison of molecular weights of keratan sulphate estimated by gel filtration and calculated from mannose content

Estimation of molecular weights by gel filtration was achieved on a calibrated Bio-Gel P-100 column (1.28 cm × 116 cm) after treating the *N*-acetylated and amidated keratan sulphate with alkali-borohydride under the conditions as described in Table 3, except cornea keratan sulphate which was digested exhaustively with papain and Pronase. Molecular weights were also calculated from the molar ratios of galactose to mannose by multiplying by the molecular weight of the repeating disaccharide monosulphate unit.

Source	Molecular weight of keratan sulphate		
	By gel filtration	From molar ratios	
		Galactose/mannose	Glucosamine/mannose
Costal cartilage of			
Children	10500	13200	10500
Young adult with Marfan syndrome	10400	12400	10200
Old human	11000	7900	6500
Pig nucleus pulposus	11900	12000	9300
Bovine cornea	11900	5400	5100

per chain, whereas each chain of the keratan sulphate I contains two mannose residues.

Solubility and degree of depolymerization of desulphated keratan sulphate

After desulphation of the nucleus pulposus keratan sulphate in methanolic HCl (see the Experimental section) the solution was evaporated to 2 ml, adjusted to a concentration of 1 mg/ml and kept for up to 10 days at 4°C with no precipitation. The salts in the solution were then removed by passing through an ion-exchange column consisting of two layers of AG50-X8 resin (H⁺ form; 10 ml) on the top of AG1-X8 resin (formate form, 10 ml) eluting with water (60 ml). The solution was brought to a concentration of 1 mg/ml and again no precipitate was observed for 10 days at 4°C. The product was freeze-dried, yielding a white residue (yield 81.4%). Analysis for sulphate was negative. However, only 87.3% of the freeze-dried material was soluble in water at a concentration of 1 mg/ml, even after stirring for 2 days at room temperature. The water-insoluble material was also insoluble both in dilute acid (0.1 M-HCl) and in alkaline (0.1 M-NaOH) solutions, but soluble in strong acid (4 M-HCl), formamide and dimethyl sulphoxide.

The extent of depolymerization of the water-soluble and insoluble fractions was examined by gel filtration (with formamide as eluent) on a Bio-Gel P-100 column (1 cm × 100 cm), monitoring the effluent by the anthrone reaction. The water-soluble fraction showed a more retarded and broader elution pattern than that of intact keratan sulphate. It had a small peak at the void volume, whereas all of the water-insoluble fraction was eluted at the void volume (see Fig. 1). There was no significant difference in the compositional analysis of water-soluble and water-insoluble fractions. The solubilization of the water-

Fig. 1. Gel filtration on a Bio-Gel P-100 column of native and desulphated keratan sulphates of pig nucleus pulposus in formamide

The samples were applied to the column (1 cm × 100 cm) and eluted with formamide; the effluent was assayed by the anthrone method. Δ , Native keratan sulphate; $\bullet$, water-insoluble and $\circ$, water-soluble fractions on mild acid-catalysis; $\square$, base-catalysed desulphation product; V_0 , void volume.

insoluble fraction by formamide apparently did not result in complete disaggregation of the molecules.

The base-catalysed desulphation product, after the removal of the organic solvents by vacuum distillation, was passed through an anion-exchange column of AG1-X8 resin (formate form; 15 ml) which was eluted with water. The effluent was brought to a concentration of 1 mg of the starting material/ml

Table 5. *Methylation products of keratan sulphate from different sources*

Methylation was achieved by Hakomori procedure (1964) in dimethyl sulphoxide with three additions of methylsulphinyl-carbanion and methyl iodide alternatively, followed by two additions of Purdie's reagents (see Purdie & Irvine, 1903).

Source of keratan sulphate ...	Yield or content (%)						
	Costal cartilage			Pig nucleus pulposus			Bovine cornea
	Children	Young adult with Marfan syndrome	Old human	Native	AcNeu removed	Sulphate removed	
Yield*	73.2	84.2	81.3	79.4	83.3	78.3	72.1
Methoxyl content†	27.8	26.6	26.3	26.1	26.5	37.9	26.7
Sulphate	16.9	17.5	18.5	18.0	17.2	0.8	17.7

* Overall yield (see the Experimental section).

† The theoretical value is 28.8% for the repeating disaccharide monosulphate and 41.1% for the repeating disaccharide without the sulphate group.

and no precipitate was observed up to 10 days at 4°C. The product, recovered by freeze-drying and yielding a white residue (yield 80.2%), was readily soluble in water, and analysis for sulphate was negative. Gel filtration of the product was carried out in the same Bio-Gel P-100 column (with formamide as eluent) as described above, and the elution pattern was even more broad and retarded ($K_d = 0.72$) than that of the water-soluble fraction of the acid-catalysed desulphation product (Fig. 1). The gel filtration of the water-soluble fractions from both acid-catalysed and base-catalysed desulphation, and the intact keratan sulphate through the same column (with aq. 0.5M-NaCl as eluent) resulted in similar elution patterns with K_d values of 0.14, 0.31 and 0.87 for the intact keratan sulphate, acid-catalysed and base-catalysed desulphation products respectively. The reducing-sugar value of each fraction determined by ferricyanide was also in the same order as the K_d value. The difference of the K_d value between the intact and the desulphated keratan sulphates is probably a reflection of changes in the molecular sizes rather than a change in the hydrodynamic properties brought about by the desulphation. The degradation is apparently much more extensive in the base-catalysed than in the acid-catalysed product and the latter was used in the methylation studies.

Methylation analysis

Methylation of keratan sulphate was achieved by the Hakomori procedure in dimethyl sulphoxide, with three additions of methylsulphinyl carbanion and methyl iodide, alternatively, followed by two additions of Purdie's reagents. Further repetition of both methylation methods did not increase significantly the methoxyl content. The overall yield of the methylation products ranged from 72 to 84% and their methoxyl contents were close to the theoretical value (Table 5). Desulphation apparently did not

occur during the methylation procedure (Table 5). Sialyl groups survive the methylation procedure, as previously reported (Choi & Carubelli, 1968). To determine the location of sialic acid and sulphate groups in pig nucleus pulposus keratan sulphate, methylation was carried out before and after the removal of the respective groups.

The identity of the partially methylated sugars in the hydrolysate of the permethylated keratan sulphate was established by (a) g.l.c. retention times and mass-spectral analysis after reduction and acetylation (b) by oxidation with sodium metaperiodate and in part (c) demethylation. The results are summarized in Tables 6 and 7. Table 6 represents all the peaks observed in the gas chromatogram of the neutral sugar derivatives obtained from the permethylated keratan sulphate by acid hydrolysis followed by reduction and acetylation, except two minor peaks of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylgalactitol and 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylgalactitol from nucleus pulposus keratan sulphate, each being less than 1% of the total neutral sugar derivatives. The data indeed demonstrate the complexity of keratan sulphate but permitted the following conclusions: (1) Fucose is terminal in all fractions and obviously more variable than can be accounted for by the chain length of the different fractions. (2) The sialyl groups are terminal and linked to 3-positions of galactosyl groups. (3) The heterogeneity of the chains is indicated by the presence of the mannosyl groups with at least three different substituents in the human costal-cartilage keratan sulphates and in keratan sulphate I, and two different substituents in the pig nucleus pulposus keratan sulphate. This was unexpected because the comparison of molecular weights by gel filtration and the calculated values from molar ratio galactose/mannose indicated that the keratan sulphate II of pig nucleus pulposus and costal cartilages of children and a young adult had one

Table 6. *G.l.c. analysis of O-methyl neutral sugars from the permethylated keratan sulphate from various sources, as alditol acetates*

Compounds were identified by g.l.c. retention times, mass spectra and susceptibility to sodium metaperiodate oxidation. The data represent means of at least three determinations by g.l.c. and mass-spectral analyses. Values of major peaks agree within 5%. The proportions (%) are based on the total neutral sugar alditol acetates. R_T , relative retention time.

Compound peracetate	R_T	Proportion (%)						
		Costal cartilage			Pig nucleus pulposus			Bovine cornea
		Children	Young adult with Marfan syndrome	Old human	Native	AcNeu removed	Desulphated	
2,3,4-Tri- <i>O</i> -methylfucitol	0.55	5.5	6.7	7.3	0.6	0.6	0.0	2.5
2,3,4,6-Tetra- <i>O</i> -methylgalactitol	1.00	2.7	3.2	3.6	4.2	11.3	23.3	3.9
3,4,6-Tri- <i>O</i> -methylmannitol	1.52	0.7	0.2	1.8	0.4	0.2	0.5	6.4
2,4,6-Tri- <i>O</i> -methylgalactitol	1.78	59.8	44.2	25.3	31.4	31.5	64.3	54.1
4,6-Di- <i>O</i> -methylmannitol	2.27	1.2	3.7	2.3	4.2	5.5	5.4	2.0
2,3,4-Tri- <i>O</i> -methylgalactitol	2.53	3.4	1.6	6.0	3.8	4.0	1.8	1.4
4,6-Di- <i>O</i> -methylgalactitol	2.72	0.0	0.0	0.8	3.5	1.1	1.1	0.0
2,4-Di- <i>O</i> -methylmannitol	3.51	2.3	4.5	0.9	0.0	0.0	0.0	1.6
2,4-Di- <i>O</i> -methylgalactitol	4.83	24.4	35.8	52.2	57.4	54.1	3.3	28.0

Table 7. *G.l.c. analysis of O-methyl amino sugars from the permethylated keratan sulphate from various sources, as alditol acetates*

The methods applied for the identification of compounds were as indicated in Table 6. The proportions (%) are based on the total *O*-methylated 2-deoxy-2-(*N*-methylacetamido)glucitol peracetates. No 2-amino-2-deoxygalactose derivative was observed. R_T , relative retention time to 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol. The desulphated keratan sulphate of pig nucleus pulposus had a small amount (4.5% of the total 2-amino-2-deoxyglucitol derivative) of 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol.

2-Deoxy-2-(<i>N</i> -methylacetamido)-glucitol peracetate	R_T	Proportion (%)						
		Human costal cartilage			Pig nucleus pulposus			Bovine cornea
		Children	Young adult with Marfan syndrome	Old human	Native	AcNeu removed	Desulphated	
3,6-Di- <i>O</i> -methyl-	1.77	24.6	23.9	17.3	50.9	51.7	91.3	24.7
3- <i>O</i> -Methyl-	4.00	75.4	76.1	82.7	49.1	48.3	4.2	73.3

mannose residue per chain, one to two in the costal cartilage keratan sulphate of old human and two in keratan sulphate I. Even though satisfactory g.l.c. retention times and mass spectra were obtained for the assignment of peaks in the gas chromatogram for the mannose derivatives, it was thought that further confirmation of the assignment was desirable with keratan sulphate fractions with a higher mannose content, which have been obtained from an endo- β -galactosidase digest of keratan-sulphate. (Y. Kikuchi, H. Choi and K. Meyer, unpublished results). (4) The yield of tetra-*O*-methylgalactitol peracetates is an indicator of the extent of branching of chains with galactose at non-reducing ends. The data in Table 6 demonstrate that the keratan sulphate preparation from pig nucleus pulposus appears to be composed of highly branched chains and where most of the

branches terminate with *N*-acetylneuraminic acid. The value of 11.3% for the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol from the sialic acid-free keratan sulphate of pig nucleus pulposus (Table 6) is equivalent to 3.2 terminal galactose units per chain assuming a molecular weight of 12000 for the native keratan sulphate. Further, the molar ratio of sialic acid to mannose from the compositional analysis of the native keratan sulphate was 3.9 (Table 1). It is also interesting that whereas the terminal galactose of the native keratan sulphate after neuraminidase digestion was resistant to β -exo- β -galactosidase from *Escherichia coli*, it was susceptible to the exo- β -galactosidase preparation from *Coccobacillus* (Choi & Meyer, 1975) yielding free galactose in an amount that was 19.9% of total galactose and equivalent to 4.8 terminal galac-

tose residues per chain assuming a mol.wt. of 12000 for native keratan sulphate. Overall, each chain of the pig nucleus pulposus keratan sulphate appears to have three to five branches per chain. Assuming structural analogies between pig nucleus pulposus keratan sulphate and that of costal cartilages, the presence of large amounts of the *N*-acetylneuraminic acid and fucose in the keratan sulphate of the costal cartilage of children (Table 1) appears to indicate that the keratan sulphate has even more branches here than that of pig nucleus pulposus whereas that in bovine cornea has no branching. The branching in human costal-cartilage keratan sulphates decreases with age.

Also noteworthy was the isolation of *N*-acetylneuraminylgalactose from bovine nasal septum keratan sulphate after alkali treatment, which was interpreted as an alkaline elimination from position-3 of *N*-acetylgalactosamine at the keratan sulphate-peptide linkage (Hopwood & Robinson, 1974), and the isolation of the disaccharide galactosyl(1→3)-*N*-acetylgalactosamine from a mixture after partial acid hydrolysis of bovine nasal septum keratan sulphate (Kieras, 1974). A similar study was made with the pig nucleus pulposus keratan sulphate by treating it (after removal of the sialic acid with neuraminidase) with alkali (0.5M-NaOH) for 10 days at 4°C. Analysis of the product by the sugar analyser yielded free galactose in an amount that was 2.8% of total galactose and equivalent to 0.7 residues per chain. The result appears to support the proposal that the substituent of the C-3 position of *N*-acetylgalactosamine at the keratan sulphate-protein linkage is *N*-acetylneuraminylgalactose. (5) The methylation analysis of the keratan sulphates of human costal cartilages demonstrates a subtle increase of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol with age (Table 6), whereas the compositional analysis of the native keratan sulphate showed a marked decrease in *N*-acetylneuraminic acid with age (Table 1). This appears to indicate that the keratan sulphate of human costal cartilage *in vivo* loses some of the labile groups with aging. (6) The almost complete conversion of 1,4,5,6-tetra-*O*-acetyl-2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glucitol to the 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl analogue (Table 7) together with almost quantitative conversion of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol to 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol (Table 6) by desulphation before methylation proves that practically all sulphate groups in keratan sulphate are located on the 6-position of both parent sugars. (Unfortunately it has not been possible to obtain a methylated derivative of the galactosaminyl groups, since the latter are totally destroyed during methylation and subsequent hydrolysis.) One of the most interesting findings during this investigation was the variation of the sulphate groups according to age and source.

The overall sulphation is higher in old than in young costal keratan sulphate and the increased sulphation with age is mainly on the galactose residues. In one case of Marfan syndrome, the values fell in between those obtained from children and those obtained from old cartilages, suggesting that the pattern of the sulphation corresponds to the age and not to the genetically determined pathology of this disease. The overall sulphation in pig nucleus pulposus keratan sulphate is slightly higher than that in old human costal keratan sulphate (Table 1). However, the sulphation of the glucosamine residue is much lower than that of any of the costal keratan sulphate studied (Table 7), whereas the sulphation of the galactose moiety is slightly higher than that of old human cartilage keratan sulphate (Table 6). The extent and pattern of sulphation in bovine cornea keratan sulphate is intermediate between that found in the keratan sulphates of children and young adults.

Although the results reported in the present paper confirm and significantly extend earlier findings, they still do not permit an unequivocal representation of the structures of carbohydrate chains of keratan sulphates. In view of the polydispersity and heterogeneity of the carbohydrate chains, their multiple and variable substituents and their changes with age, it is doubtful whether this ideal can be totally achieved. The sequence of the variable chains in the proteoglycans, and the sequence of the sulphate groups in the various chains and their changes with aging will remain a challenge for which no ready solution appears in sight. We know from the results of Hirano & Meyer (1973) that on digestion with the endo- β -D-galactosidase the sulphate groups are not uniformly distributed along the chains, but are bunched toward the reducing ends, thus preventing or at least inhibiting further enzymic degradation by endo- or exo-glycosidase. The problems listed above and many others make an attempt to study the biosynthesis of these compounds a foolhardy undertaking.

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