Degradation of articular cartilage keratan sulphates using hydrazinolysis and nitrous acid

Environment of fucose residues

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Alkaline borohydride-reduced keratan sulphate (KS) chains from bovine articular cartilage (6–8-year-old animals) were fragmented by an anhydrous hydrazine/nitrous acid procedure, previously used on KS by Hopwood & Elliott to isolate the major disaccharides from the poly-*N*-acetyl-lactosamine repeat sequence [Hopwood & Elliott (1983) Carbohydr. Res. 117, 263–274]. The resulting oligosaccharides were reduced with NaB³H₄ or NaBH₄ and subjected to ion-exchange chromatography on a Nucleosil 5SB column. In addition to the major disaccharides, two fucose-containing oligosaccharides were examined by high-field ¹H n.m.r. spectroscopy, and shown to have the following structures (where AnManOH is 2,5-anhydro-D-mannitol):

It is evident that the presence of fucose protects the *N*-acetylglucosamine residue from de-*N*-acetylation, and therefore fragments are produced which preserve the immediate environment of the fucose residue. It may be of biosynthetic significance that these two oligosaccharides contain an unsulphated galactose on the non-reducing side of the fucose residue. The hydrazine/nitrous acid/NaB³H₄ method followed by h.p.l.c. provides a sensitive fingerprinting technique for the assay of KS composition and sub-populations.

INTRODUCTION

Keratan sulphate (KS), a glycosaminoglycan, was first isolated from bovine cornea by Meyer *et al.* (1953) and has subsequently been shown to be present in many other tissues. KS has been classified (Meyer, 1970) as two separate forms, KS-I from the cornea and KS-II from skeletal tissues such as cartilage, the two types differing in their mode of linkage to protein. Recently, Nieduszyński *et al.* (1990) have proposed a further sub-classification of KS-II into two types. KS-II-A from articular and bovine intervertebral-disc cartilage contains two structural features, namely α (1–3)-fucose and α (2–6)-linked *N*-acetylneuraminic acid residues, that are absent from KS-II-B from tracheal and nasal-septum cartilage. Indeed, a sub-population of articular cartilage KS chains capped with α (2–6)-linked *N*-acetylneuraminic acid residues has been isolated by Tai *et al.* (1992) by affinity chromatography using the lectin from *Sambucus nigra*.

The structure of KS is known to be based upon a repeating *N*-acetyl-lactosamine sequence of -3Gal β 1-4GlcNAc β 1- which is usually sulphated on C-6 of *N*-acetylglucosamine, but further sulphate groups may be present on C-6 of galactose (Bhavanandan & Meyer, 1968). The detailed structure of KS is still poorly understood, but recent studies by Dickenson *et al.*

(1990, 1991, 1992) using a combination of enzymic fragmentation with the KS specific enzyme keratanase and high-field ¹H n.m.r. spectroscopy have elucidated the nature of various capping sequences and linkage regions. Recent studies by Tai *et al.* (1991) have demonstrated that the fucose content in a preparation of bovine articular cartilage KS increased linearly with relative molecular mass and those authors concluded that the fucose residues are located within the sulphated poly-*N*-acetyl-lactosamine repeat sequence.

A combination of hydrazinolysis and nitrous acid deamination has long been recognized as a powerful method for the study of polysaccharides containing sugars with *N*-acetyl groups on C-2. Treatment with anhydrous hydrazine removes the acetyl groups to leave a free amine group which can then be removed by nitrous acid, causing a rearrangement of the sugar ring and cleavage of the *O*-glycosidic linkage on the reducing side (Fig. 1). In the case of KS, this produces a 2,5-anhydro-D-mannose residue into which a radiolabel can be introduced by reducing it to 2,5-anhydro-D-[1-³H]mannitol (AnMan-OH) by using sodium borotr[³H]₄hydride.

In this study, KS-derived fucose-containing oligosaccharides have been produced by a hydrazinolysis/nitrous acid procedure, fractionated by ion-exchange chromatography, and subsequently characterized by ¹H n.m.r. spectroscopy.

Abbreviations used: KS, keratan sulphate; g.p.c., gel-permeation chromatography; Gal(6S), β -D-galactose 6-*O*-sulphate; AnMan-OH, 2,5-anhydro-D-mannitol; AnMan-OH(6S), 2,5-anhydro-D-mannitol 6-*O*-sulphate; HOHAHA, homonuclear Hartmann-Hahn; 2D, two-dimensional n.m.r. spectroscopy; COSY, 2D homonuclear chemical-shift correlation spectroscopy.

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Fig. 1. De-*N*-acetylation of KS using anhydrous hydrazine followed by nitrous acid deaminative cleavage of glucosamine glycosidic linkages

EXPERIMENTAL

Materials

Chemicals and enzymes used in this study are as described previously (Dickenson *et al.*, 1990). Anhydrous hydrazine and hydrazine sulphate were from Aldrich (Gillingham, Dorset, U.K.).

Isolation and purification of KS chains

Peptido-KS fragments from bovine femoral head cartilage (6–8-year-old animal) were prepared essentially by the method described by Dickenson *et al.* (1990). KS chains were subsequently prepared by alkaline borohydride reduction (Carlson, 1968) followed by chondroitin ABC lyase digestion and gel-permeation chromatography (g.p.c.) on a column of Sephadex G-50 (82 cm × 1.5 cm) eluted with 0.15 M-NaCl. After dialysis against water, the KS chains were recovered by lyophilization and then purified further from any remaining *O*-linked oligosaccharides by ion-exchange chromatography on a Pharmacia

Mono-Q HR 10/10 column eluted with a linear gradient of 0–0.5 M-LiClO₄/10 mM-piperazine, pH 5.0.

De-*N*-acetylation of KS chains

De-*N*-acetylation was performed essentially by the method of Hopwood & Elliott (1983). KS chains (30 mg) were placed in a 5 ml reacti-vial with 225 mg of hydrazine sulphate as catalyst. Anhydrous hydrazine (3 ml) was added and the mixture was heated for 4 h at 97–98 °C. All work involving anhydrous hydrazine was performed in a glove box under dry nitrogen. The reaction was stopped by the careful addition of 2 ml of water. After extensive dialysis against water the de-*N*-acetylated KS was recovered by lyophilization.

Deamination of de-*N*-acetylated KS

Deamination was performed using the method of Lindahl *et al.* (1973). To 29 mg of de-*N*-acetylated KS was added 2.9 ml of 3.9 M-NaNO₂/0.28 M-MeCO₂H, pH 4.1, and the mixture was left for 2 h at room temperature. The reaction was terminated by cooling the mixture on ice and adjusting to pH 7.5 with 2 M-NH₄OH.

Alkaline borohydride/borotritide reduction

The oligosaccharide mixture (100 μl; containing approx. 1 mg of oligosaccharides) was withdrawn and added to 100 mCi of NaB³H₄ (190.2 mCi/mg). This mixture was left for 1 h at room temperature, after which a solution of 4.85 mg of NaBH₄ in 40 μl of water, pH 7.5, was added to raise the borohydride concentration to approximately 1 M. The mixture was left for a further 2 h and then the reaction was terminated by cooling the mixture on ice and neutralizing with 4 M-acetic acid. The oligosaccharides were desalted on a Bio-Gel P2 column (11.2 cm × 1 cm) eluted with water at 50 °C.

The remainder of the deaminated KS was reduced by the addition of 113 mg of NaBH₄ and the mixture was left for 3 h at room temperature, after which the reaction was terminated as above. The oligosaccharides were desalted in three runs on a Bio-Gel P2 column (45 cm × 0.9 cm) eluted with water at 50 °C and monitored by refractive index.

Ion-exchange chromatography

H.p.l.c. was performed on a Bio-Rad series 700 HRLC titanium gradient system with u.v. and conductivity monitors. Approx. 300 μg of the radiolabelled oligosaccharides was applied to a Nucleosil 5SB column equilibrated in 2 mM-LiClO₄, pH 5, and eluted with a linear gradient of 0–0.25 M-LiClO₄, pH 5. Fractions were monitored for radiolabel using a Packard Tri-Carb 300 scintillation counter (Fig. 2); 0.5 ml aliquots were taken and diluted to 5 ml with Ecoscint A.

The reduced unlabelled oligosaccharides (19 mg) were chromatographed in two separate runs (9.5 mg each) on the Nucleosil 5SB column under the same conditions as above and the eluate was monitored at 216 nm. Fractions were pooled with reference to the radioactivity profile (Fig. 2) to give fractions 1 and 2, which were subsequently examined by ¹H n.m.r. spectroscopy. Fraction 2 was found to be a single compound and will be referred to as oligosaccharide (II). Fraction 1 was found to be a mixture of several components and these were separated by rechromatographing the sample on a Nucleosil 5SB column with a shallower gradient of 0–100 mM-LiClO₄, pH 5. Oligosaccharide (I) was pooled as shown in Fig. 2 (inset).

N.m.r. spectroscopy

Samples were buffered to pH 7 with phosphate, referenced with sodium 3-trimethylsilyl[²H₃]propionate as internal standard

Fig. 2. High-performance anion-exchange chromatography profile of the ^3H -labelled oligosaccharides produced by hydrazinolysis/nitrous acid/sodium borohydride treatment of articular cartilage KS

The Nucleosil 5SB column (25 cm $\times$ 10 mm) was eluted at a flow rate of 2 ml/min. Fractions (2 ml) were assayed for radioactivity using a Packard Tri-Carb 300 scintillation counter. The gradient programme was as follows: 15 min of buffer A (2 mM- LiClO_4 , pH 5.0) and then 180 min of 0–100% buffer B (0.25 M- LiClO_4 , pH 5.0). Fractions 70–75 (Fraction 1) and fractions 118–120 (Fraction 2) were pooled as shown. Inset: re-chromatography of fractions 70–75. The gradient programme was as follows: 10 min of buffer A (2 mM- LiClO_4 , pH 5.0) and then 180 min of 0–100% buffer B (100 mM- LiClO_4 , pH 5.0). Fractions 78–82 were pooled as oligosaccharide (I).

and dissolved in 0.5 ml of 99.96% $^2\text{H}_2\text{O}$ after microfiltration and several previous exchanges with 99.8% $^2\text{H}_2\text{O}$ and one using 99.96% $^2\text{H}_2\text{O}$. ^1H n.m.r. spectra and two-dimensional homonuclear Hartmann–Hahn (HOHAHA) measurements were determined at 60 $^\circ\text{C}$ using Bruker AMX600 & AM500 spectrometers. COSY-45 and one-step relayed homonuclear chemical-shift correlation (COSY) two-dimensional measurements were performed at 60 $^\circ\text{C}$ on a JEOL GSX400 spectrometer. The 400 MHz data were reprocessed for presentation using the computer program NMR2 (New Methods Research, Syracuse, NY, U.S.A.).

RESULTS

The findings of Hopwood & Elliott (1983) suggested that the major oligosaccharides produced from KS would be the mono- and di-sulphated disaccharides consisting of galactose linked $\beta(1\text{--}4)$ to AnMan-OH. Examination of the ion-exchange profile (Fig. 2) clearly shows two major peaks, at approx. 48 and 87 min, which have been identified by ^1H n.m.r. spectroscopy (G. M. Brown, T. N. Huckerby & I. A. Nieduszynski, unpublished work) as the disaccharides Gal $\beta(1\text{--}4)$ AnMan-OH(6S) and Gal(6S) $\beta(1\text{--}4)$ AnMan-OH(6S) respectively. In the case of incomplete de-*N*-acetylation, a series of oligosaccharides containing an even number of sugar residues would be produced which vary both in the extent and the location of ester sulphate groups.

N.m.r. spectroscopic analysis of the major oligosaccharides produced in this study identified two oligosaccharides, compounds (I) and (II), giving signals characteristic of $\alpha(1\text{--}3)$ -linked fucose. From their relative elution positions on the Nucleosil 5SB column it is also clear that oligosaccharide (II) possesses a higher charge density than does oligosaccharide (I). The ^1H n.m.r. spectra of these two oligosaccharides (Figs. 3a and 3b) clearly demonstrate the presence of four discrete anomeric resonances which, together with the knowledge that AnMan-OH will occupy

Fig. 3. Partial 600 MHz ^1H n.m.r. spectra at 60 $^\circ\text{C}$ for (a) oligosaccharide (I) and (b) oligosaccharide (II)

the reducing terminus of any oligosaccharide produced from the repeat sequence of KS, suggests that both of these oligosaccharides are pentasaccharides. Chemical-shift assignments were made using one- and two-dimensional n.m.r. methods and by comparison with previously assigned spectra from related molecules. An almost complete set of assignments at 60 $^\circ\text{C}$ and partial assignments (for clearly resolved resonances) at 23 $^\circ\text{C}$ are given in Table 1.

Examination of the proton spectra of both oligosaccharides (I) and (II) clearly shows an anomeric resonance at 4.801 p.p.m. in the spectrum of oligosaccharide (I) and at 4.818 p.p.m. in that of oligosaccharide (II), characteristic of an internal *N*-acetylglucosamine residue. The value of the coupling constant and the observation of 'virtual coupling' often seen because of interaction with strongly coupled *N*-acetylglucosamine ring protons confirms this assignment. Complete signal assignments can be made for this residue by using 2D data showing that, for oligosaccharides (I) and (II), the H-6 and H-6' methylene protons occur at ~ 4.37 p.p.m., thereby indicating that this residue is sulphated on C-6 in both oligosaccharides. The presence of an unsubstituted galactose residue in oligosaccharides (I) and (II) is indicated by the readily identifiable H-4 resonance at 3.936 and 3.938 p.p.m. respectively. COSY data demonstrate that this signal connects to

Table 1. Spectral data for oligosaccharides (I) and (II)

Chemical shifts are in p.p.m. from internal sodium 3-trimethylsilyl[$^2\text{H}_6$]propionate and unless indicated are from first-order analyses of signals.

	Oligosaccharide (I)		Oligosaccharide (II)	
	60 °C	23 °C	60 °C	23 °C
Fuc				
H-1	5.136	5.142	5.136	5.142
H-2	3.709 ^a		3.705 ^a	
H-3	3.914		3.917	
H-4	3.799 ^a		3.798 ^c	
H-5	4.763		4.765	
Me	1.191	1.187	1.191	1.186
Gal ¹				
H-1	4.531	4.539	4.556	4.561
H-2	3.495	3.498	3.493	3.504
H-3	3.672		3.673	
H-4	3.936		3.938	
H-5				
H-6				
H-6'				
GlcNAc				
H-1	4.801		4.818 ^d	4.849
H-2	3.965 ^a		3.973 ^a	
H-3	~ 3.96 ^b		~ 3.95 ^b	
H-4	3.982 ^a		4.002 ^c	
H-5	3.841 ^a		3.820	
H-6	4.370 ^a	4.375	4.372	~ 4.40/4.36 ^e
H-6'	4.370 ^a	4.375	4.372	~ 4.40/4.36 ^e
NAc	2.031	2.031	2.031	2.029
Gal ²				
H-1	4.478	4.471	4.503	4.498
H-2	3.618		3.615 ^a	
H-3	3.724 ^b		3.748 ^a	
H-4	4.167	4.177	4.207 ^a	
H-5			3.970 ^c	
H-6			~ 4.21–4.24	
H-6'			~ 4.21–4.24	
AnMan-OH				
H-1	3.708 ^b /3.79 ^b		3.720 ^a /3.782 ^c	
H-1'	3.708 ^b /3.79 ^b		3.720 ^a /3.782 ^c	
H-2	3.993 ^a		4.005 ^a	
H-3	4.254 ^a		4.288 ^a	
H-4			4.210 ^a	
H-5			~ 4.28 ^c	
H-6			~ 4.2 ^c	
H-6'			~ 4.2 ^c	

^a Chemical shift taken from COSY-45 spectrum.^b Chemical shift taken from relayed-COSY spectrum.^c Chemical shift taken from 2D-HOHAHA spectrum.^d Signal shows 'virtual coupling' due to very strong couplings between protons 2, 3 and 4.^e Strongly coupled ABX system.

the anomeric signal at 4.531 p.p.m. and 4.556 p.p.m. respectively for oligosaccharides (I) and (II), which can therefore be assigned as H-1 of a terminal galactose residue. The chemical shifts of H-1–H-4 in this residue compare closely to those found by Strecker *et al.* (1987) and Dickenson *et al.* (1991) for an unsulphated terminal galactose linked to a sulphated *N*-acetylglucosamine residue.

Examination of the 2D COSY-45 spectra (Figs 4a and 4b) and HOHAHA spectra (Figs. 5a and 5b) for these oligosaccharides clearly shows the presence of a non-equivalent methylene pair at

~ 3.7–3.8 p.p.m. in both cases which can be assigned as H-1, H-1' of AnMan-OH. COSY connections are visible to H-2 at 3.993 and 4.005 p.p.m. for oligosaccharides (I) and (II) respectively, which in turn connect to a complex envelope of signals at ~ 4.2–4.3 p.p.m. containing signals from the remainder of the AnMan-OH protons. For oligosaccharide (I), although a complex very tightly coupled set of signals was seen in the range 4.2–4.3 p.p.m., it was not possible to follow either COSY or HOHAHA connections beyond H-3 because the spin system was too strongly second-order in character.

Fig. 4. Partial 400 MHz COSY-45 spectra at 60 °C

(a) Oligosaccharide (I). The spectral width was 2183 Hz and 48 acquisitions for each of 1024 t_1 increments were sampled into 1024 complex points. (b) Oligosaccharide (II). The spectral width was 2250 Hz and 64 acquisitions for each of 512 t_1 increments were sampled into 512 complex points. Both 2D data sets were transferred to a VAX 4000 computer and then processed for presentation and detailed analysis using the software package NMR2 (New Methods Research Inc., Syracuse, NY, U.S.A.). The arrays were zero-filled to 2048 $\times$ 2048 complex points and transformed in each dimension after application of a 2% offset sinebell window function.

As mentioned previously, the spectra for oligosaccharides (I) and (II) display clear signals at 5.136 and 1.191 p.p.m. which are readily recognizable as the H(1) and H(6) resonances respectively of fucose-linked $\alpha(1-3)$ (Vliegthart *et al.*, 1983). Complete chemical-shift assignments for the fucose residue (Table 1) compare closely to those found by Huckerby *et al.* (1991) for the $\alpha(1-3)$ -linked fucose contained within intact KS chains. In the KS poly-*N*-acetyl-lactosamine repeat sequence there are no

Fig. 5. Partial 2D-HOHAHA spectra at 60 °C for oligosaccharides (I) and (II)

(a) 500 MHz spectrum for oligosaccharide (I). The spectral width was 2183 Hz and 56 acquisitions for each of 440 t_1 increments were sampled into 1024 complex points. The mixing time was 120 ms. After zero-filling to 1024 $\times$ 1024 complex points the spectrum was processed in the phase-sensitive mode using a $\pi/3$ offset (sinebell)² window function. (b) Spectrum at 600 MHz for oligosaccharide (II). The spectral width was 3623 Hz, and 48 acquisitions for each of 512 t_1 increments were sampled into 1024 complex points. The mixing time was 120 ms. After zero-filling to 2048 $\times$ 2048 complex points, the spectrum was processed in the phase-sensitive mode using a $\pi/3$ offset (sinebell)² window function.

available unsubstituted galactose 3-positions and therefore the fucose residue must be attached to *N*-acetylglucosamine (Tai *et al.*, 1991). For oligosaccharides (I) and (II), evidence that the fucose residue is in both cases linked to *N*-acetylglucosamine and not to AnMan-OH comes from two sources. First, the chemical-shift positions of the AnMan-OH ring protons are closely comparable to those found by Huckerby *et al.* (1985, 1986) for a terminal AnMan-OH residue sulphated on C-6 and substituted only on C-4. Secondly, the *N*-acetylglucosamine H-3 and H-4

proton chemical shifts are perturbed downfield by 0.2–0.3 p.p.m. from those for a sulphated internal *N*-acetylglucosamine which is substituted only (by a galactose residue) on C-4 (Huckerby *et al.*, 1990). This indicates further substitution of the ring, on C-3.

The remaining anomeric resonance in the spectrum of oligosaccharides (I) and (II) at 4.478 and 4.503 p.p.m. respectively can therefore be assigned to H-1 of a galactose residue located between the *N*-acetylglucosamine and the AnMan-OH residues. COSY-45 connections can be made as far as H-4 at 4.167 and 4.207 p.p.m. for (I) and (II) respectively, showing that this residue is indeed linked at C-4 (Huckerby *et al.*, 1990). It is not possible to observe connections on the COSY spectrum for the ring beyond H-4 because $J(4,5)$ is extremely small in this sugar. However, both the COSY and 2D HOHAHA spectra (Figs. 4*b* and 5*b*) of oligosaccharide (II) clearly show another connection from a resonance at ~ 3.97 p.p.m. to one or more protons at ~ 4.21 – 4.24 p.p.m. These shift positions are characteristic of H-5 and H-6, H-6' respectively of an internal galactose which is sulphated at C-6 (Cockin *et al.*, 1986). This connection is absent from the 2D HOHAHA spectrum of oligosaccharide (I), thus showing that this residue is unsulphated in this oligosaccharide. This constitutes the only major spectral difference between the two pentasaccharides.

The following structures can therefore be assigned to oligosaccharides (I) and (II):

It is interesting to note that the chemical-shift assignments for the fucose residue are practically identical for both oligosaccharides, indicating that it is relatively insensitive to sulphation within the galactose residue located on the reducing side of the fucosylated *N*-acetylglucosamine. Confirmation of these structures was made via ^{13}C n.m.r. measurements (results not shown) which clearly show the presence of three and two unsulphated methylene resonances at ~ 64 – 67 p.p.m. for oligosaccharides (I) and (II) respectively, together with a set of 6-*O*-sulphated AnMan-OH chemical-shift positions closely similar to those observed by Huckerby *et al.* (1985, 1986).

DISCUSSION

The object of this study was to isolate and characterize fucose-containing oligosaccharides from KS. The fucose content of articular cartilage KS tends to be low, e.g. at the 1–2% level, and therefore it is advantageous to restrict the fucose to the minimum number of oligosaccharides. The enzyme, keratanase, an endo- β -galactosidase (Nakazawa & Suzuki, 1975), is known to cleave internal $\beta(1\text{--}4)$ galactosidic bonds in which unsulphated galactose residues participate provided that the *N*-acetylglucosamine residue on the non-reducing side is sulphated. Digestion of bovine intervertebral disc KS by keratanase (J. M. Dickenson, T. N.

Huckerby & I. A. Nieduszynski, unpublished work) resulted in the fucose residue being distributed amongst many large keratanase-resistant oligosaccharides which were of inadequate yield and of too great a complexity for structural elucidation. The hydrazinolysis/nitrous acid method used by Hopwood & Elliott (1983) would appear to be preferable to keratanase because fragmentation is independent of the pattern of sulphation.

The structures of the two oligosaccharides characterized in this work confirm that fucose in articular cartilage KS occurs substituted on to the main poly-*N*-acetyl-lactosamine repeat sequence of the chain (Tai *et al.*, 1991). It was fortuitous that the presence of fucose protected the *N*-acetylglucosamine residue from deacetylation, because the larger fragments obtained have preserved the immediate sulphation environment.

All of the major oligosaccharides produced have been examined by ^1H n.m.r. spectroscopy and fucose has been detected in only these two oligosaccharides. Furthermore, the fucose content as estimated by calculating ratios of the radiolabel incorporated in these two oligosaccharides to that in the two disaccharides is very similar to the gross fucose content as estimated by ^1H n.m.r. spectroscopy (i.e. 1–2%). Therefore it seems likely that the great majority of fucose residues occur in these two environments. It is of interest to note that the fucose is directly substituted on a sulphated GlcNAc and that in both cases the galactose on the non-reducing side is unsulphated. It seems probable that the latter observation is of biosynthetic interest, as it may reflect the substrate specificities of either the fucosyltransferase or of the galactose sulphotransferase. Both sulphation and fucosylation are known to occur in the same sub-compartment of the Golgi apparatus but it is not yet known which occurs first. Further studies will be required to elucidate whether the fucose, as in oligosaccharide (I), has any influence in reducing the susceptibility of the unsulphated galactose residues in KS to cleavage by keratanase.

Emphasis has been placed in this work upon the fucose residues in KS because they occur in articular, but not in non-articular, skeletal KS species. It is interesting to note that the fucose residue occurs in the same environment as the Lewis X antigen and SSEA-1 (a carbohydrate differentiation antigen) (Feizi, 1985; Hounsell, 1987), although it is clear from the structure that these antigens may be masked by sulphation in KS (Tang *et al.*, 1986; Feizi, 1989). Indeed, it is possible that subsequent sulphation masks antigens present at early stages of tissue development. Fucose levels in KS may have a further significance if fucosylation masks epitopes recognized by antibodies used for the quantification of KS species in clinical immunoassays. It is unfortunate that at present no satisfactory antibody or lectin probes have been found for the detection of fucose in KS.

The anhydrous hydrazine/nitrous acid/ NaB^3H_4 fingerprinting method examined here would appear to have scope, if further developed, for the study of the gross chemical compositions of very small amounts of KSs [e.g. cell-surface KS-proteoglycans (Sorrell *et al.*, 1990)]. The sensitivity and ability to determine sulphation levels have previously been emphasized by Hopwood & Elliott (1983). This investigation indicates that fucosylation levels may also be determined, and further studies of the sialic acid-containing oligosaccharides should provide a means of detecting sialic acid levels and may even permit estimates of number-average relative molecular masses to be made.

The hydrazinolysis/nitrous acid degradative method has the advantage over enzymic degradations using keratanase and keratanase II (Nakazawa *et al.*, 1989) that the cleavage site is independent of the sulphation pattern. As a result any non-reducing chain cap characteristic of a discrete sub-population of

chains, e.g. Tai *et al.* (1992), will occur within an unique oligosaccharide fragment, the characterization of which provides complete quantification of that sub-population. Thus the technique described here not only provides the gross chemical composition of a KS but will also facilitate the quantification of sub-populations if a chromatographic technique of adequate resolving power and reproducibility is used.

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