Multiple Non-reducing Chain Termini Isolated from Bovine Corneal Keratan Sulfates*

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Keratan sulfate-containing proteoglycans were isolated from bovine cornea (15-month-old to 3-year-old animals) and digested with the enzyme, keratanase II. The released oligosaccharides, which included non-reducing termini and repeat region oligosaccharides but not linkage regions, were reduced with alkaline borohydride and fractionated on a Spherisorb column.

These oligosaccharides were examined by 600-MHz 1H NMR spectroscopy using one- and two-dimensional methods and, in addition to some oligosaccharide alditols previously recovered from skeletal keratan sulfate, the following new capping structures were identified: NeuAcα2-6Galβ1-4GlcNAc(S)-ol, NeuAcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-6Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuAcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol, NeuGcα2-3Galβ1-4GlcNAc(S)-ol.

These structures represent seven families of capping residues, whose relative molar proportions are given in parentheses: NeuAcα(2–3)- (12%), NeuAcα(2–6)- (41%), NeuGcα(2–3)- and NeuGcα(2–6)- families (12%), Galβ1–3- (26%), GalNAcβ(1–3)- (5%), and GlcNAcβ(1–3)- (4%). It is not clear, at present, where each of these structures occurs on the bi-antennary N-linked corneal keratan sulfate chains, which themselves occur within three keratan sulfate proteoglycan species. However, examination of the relative proportions of the capping to the repeat structures and knowledge of the average molecular size suggests that the sum of these non-reducing termini represents the caps of two antennae.

The corneal stroma is mostly an extracellular matrix, which comprises collagens, proteoglycans, and matrix proteins. Its transparency is dependent upon its hydration and the orderly arrangement of the collagen fibrils. The fibrils in mammalian stroma measure 25–30 nm in diameter and have a mean interfibrillar distance of 66 nm within lamellae (1).

The proteoglycans of the corneal stroma are associated with the collagen fibrils, and they comprise the keratan sulfate (KS)1 and the chondroitin/dermatan sulfate (CS/DS) families. Electron microscopic studies have shown that four separate proteoglycan binding sites lie within the D period of collagen fibrils in rabbit cornea. The KS proteoglycans were located at the α or “step” bands and CS/DS proteoglycans were found at the d or “gap zone.” A similar pattern was obtained in bovine cornea (2). On the basis of such studies, Scott has proposed a model (3) for proteoglycan-collagen interactions in cornea in which duplexed glycosaminoglycan chains (both double-stranded DS and KS) may bridge collagen fibrils and thus ensure the precise interfibrillar distances vital for corneal transparency.

The major CS/DS proteoglycan of cornea is decorin (4). This is a member of a group of small interstitial proteoglycans, which includes biglycan and fibromodulin that all interact with fibrillar collagens via their protein cores.

Recent studies of corneal KS have revealed the existence of several discrete proteoglycans. Originally two core proteins (37 and 25 kDa) were identified (5). Subsequently, two forms of the 37-kDa protein (37A and 37B) were resolved (6). Then, a cDNA clone was isolated and sequenced that coded for a chicken corneal KSAP. This protein, lumican, had a deduced molecular mass of 37 kDa and a sequence related to fibromodulin, decorin, and biglycan (7). This protein was shown to have a high homology with the bovine 37B core protein, which was thus identified as bovine lumican (8). The bovine 37A core protein has recently been sequenced and named keratocan (9).

Keratan sulfate (KS) was first isolated from the bovine corneal stroma by Meyer (10), who subsequently classified KS types (11). Corneal KS with an alkali-stable bond between N-acetylglucosamine and asparagine was called KS-I, and skel-etal KS with the alkali-labile bond between N-acetylglucosamine and asparagine was called KS-II. This skeletal KS type has been subclassified into articular, KS-II-A, and non-articular, KS-II-B (12), on the basis that the former contains α(1–5)-fucose and α(2–6)-N-acetylneuraminic acid, which are absent in the latter. Finally, a third type of KS obtained from rat brain was shown to be O-linked between mannose and serine or threonine was designated KS-III. This skeletal type has been further subclassified into articular, KS-II-A, and non-articular, KS-II-B (12), on the basis that the former contains α(1–5)-fucose and α(2–6)-N-acetylneuraminic acid, which are absent in the latter. Finally, a third type of KS obtained from rat brain was shown to be O-linked between mannose and serine or threonine (13).

The characteristic structure of KS involves a disaccharide repeat unit, -4GlcNAcβ1–3Galβ1-, which may be sulfated on C-6 of both the N-acetylgalcosamine and galactose residues. However, the detailed structures of specific KS types are probably best considered to be composed of three regions: linkage,

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1 The abbreviations used are: KS, keratan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; PG, proteoglycan; KSAP, keratan sulfate proteoglycan; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GlcNAc-ol, N-acetyl-D-glucosaminitol; (S), 6-sulfate; COSY, correlation spectroscopy; PNGase, peptide N-glycosidase.
repeat, and chain-capping.

For skeletal KS, these structures are now well understood. The linkage region of KS-II has been elucidated (14–16). The sulfation structure of the repeat region disaccharide has been described (17) and later a (1–3)-linked fucose was identified as occurring in the occasional sequences present in KS-II-A, -3Gal\(b_1\-4(Fuc\(a_1\-3)GlcNAc(S)\_b_1\- (18–20). The chain-capping region was shown to contain sialic acid (21) and now the keratanase-derived and reduced capping oligosaccharide alditols: NeuAc\(a_2\-3Gal\(b_1\-4GlcNAc(S)\_b_1\-3Gal-olandNeuAc\(a_2\-6Gal-\(b_1\-4GlcNAc(S)\_b_1\-3Gal-ol have been isolated and characterized (22, 23). Thus, it was shown that KS from non-articular cartilage was uniquely capped by a (2–3)-linked N-acetylneuraminic acid but chains from articular cartilage KS were capped with either a (2–3)- or a (2–6)-linked N-acetylneuraminic acid (24).

In corneal KS these structures are not so well understood. The linkage region is, apparently, well characterized and is of the complex type (25, 26), as shown in Structure 1.

\[
\text{\textbf{-Gal-GlcNAc-Man}_1, Fuc}
\]

\[
\text{\textbf{\_3Man-GlcNAc-GlcNAc-Asn}}
\]

\[
\text{\textbf{-Gal-GlcNAc-Man}_1}
\]

\[
\text{\textbf{STRUCTURE 1}}
\]

The repeat region (or regions) have been examined in detail (26, 27).

However, little is known about the chain caps in corneal KS. Sialic acid was found as a minor component in chain termini (28) and further studies showed that this residue was linked to the 3-position of galactose residues, in a ratio of sialic acid to mannose of 0.41 (29). Another study (26) concluded that sialic acid was located close to the linkage region.

The ultimate goal is to identify the structures and functions of the KS chains in each of the corneal KSPGs. However, in this study the structures of the keratanase II fragments derived from the non-linkage regions of the entire corneal KS chain population are examined.

**EXPERIMENTAL PROCEDURES**

Materials—Trizma (Tris base), 6-aminohexanoic acid, benzamidine HCl, 3,3\(9\),5,5\(9\)-tetramethylbenzidine, the anti-KS monoclonal antibody 5D4, goat anti-mouse IgG, and goat anti-rabbit IgG were obtained from Sigma (Poole, Dorset, United Kingdom (UK)); urea and guanidine hydrochloride from Fluka Biochemika (Glossop, Derby, UK); 1,9-dimethylmethylene blue from Koch-Light (Haverhill, Suffolk, UK); and EDTA from Aldrich Chemical Co. (Poole, Dorset, UK). The anti-decorin antibody, LF95, was provided by Dr. L. Fisher (National Institutes of Health, Bethesda, MD). Sepharose CL-6B, Q-Sepharose Fast Flow gel, and a Mono-Q HR 10/10 column were purchased from Pharmacia (Uppsala, Sweden). The Spherisorb S5 SAX column (1 cm × 25 cm) was from Phase Separations Ltd. (Deeside, Clwyd, UK).

Chondroitin ABC lyase (protease-free) (EC 4.2.2.4) and keratanase II were obtained from Seikagaku Kogyo Co. (via ICN Biochemicals Ltd., High Wycombe, Bucks., UK), keratanase (EC 3.2.1.103) from Sigma, endo-\(\beta\)-galactosidase (EC 3.2.1.103) and peptide N-glycosidase F (EC 3.2.2.18) from Boehringer Mannheim Biochemicals. Recombinant peptide N-glycosidase F was purchased from New England Biolabs. All other chemicals and reagents used were of analytical grade.

**Preparation of Corneal Keratan Sulfate Proteoglycans**—Bovine eyes (15-month-old to 3-year-old animals) were obtained from a local abat-
to and immediately cooled to 4 °C. They were processed within 2 days. The corneal stroma (with endothelium) was excised after the epithelium was scraped off. In a typical preparation, 110 g (wet weight) of corneas were obtained from 176 eyes, an average of 0.63 g/eye.

All of the extraction procedures were performed at 4 °C. The corneas (110 g) were extracted with 500 ml of 4 M guanidine HCl containing 0.05 M sodium acetate, pH 5.8, 0.1 M 6-aminohexanoic acid, 0.01 M EDTA, and 0.005 M benzamide HCl for 24 h. The extract was recovered by filtering through a nylonsheet, and the residual material was extracted twice more with 200 ml for 24 h. The three extracts were combined and dialyzed against 0.15 M NaCl, 7 M urea, 0.05 M Tris-HCl, pH 7.0.

The dialysate was chromatographed on a Q-Sepharose column (1.5 cm × 15 cm) in five runs. The unbound material was eluted with loading buffer: 0.15 M NaCl, 7 M urea, 0.05 M Tris-HCl, pH 7.0 (data not shown). The column was connected to a Bio-Rad HPLC system, and the bound material was eluted with a linear gradient of 0.15 M to 1.0 M NaCl containing 7 M urea and 0.05 M Tris-HCl, pH 7.0, within 40 min, and finally to 1.6 M within 10 min, at a flow rate of 2 ml/min. Fractions of 2 ml (1 min) were collected and analyzed with 1,9-dimethylmethylene blue (1-μl aliquots) for glycosaminoglycan (30) and by direct enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody 5D4 for KSPGs and antibody LF95 for CS/DSPGs.

Fractions containing KSPGs were pooled, dialyzed against water, and lyophilized (yield 482 mg). This sample was then dissolved in 15 ml of eluent: 0.15 M NaCl, 7 M urea, 0.05 M Tris-HCl, pH 7.0, and chromatographed on a Sepharose CL-6B column (2.5 cm × 15.5 cm) eluted at a flow rate of 25.6 ml/h. Fractions of 12.8 ml (30 min) were collected and analyzed by absorbance at 276 nm, 1,9-dimethylmethylene blue assay (2-μl aliquots), and direct ELISA using antibody 5D4 and LF95 (2-μl aliquots). Fractions 42–65, positive to the 1,9-dimethylmethylene blue assay, contained both KSPGs and CS/DSPGs as recognized by 5D4 and LF95. They were pooled and recovered after dialysis and lyophilization (yield 374 mg). The CS/DSPGs were digested with the enzyme chondroitin ABC lyase. Briefly, 150 mg of proteoglycans was dissolved in 15 ml of 0.1 M Tris acetate, pH 7.3, and incubated with chondroitin ABC lyase (0.6 units) at 37 °C for 10 h. The digestion was terminated by the addition of 60 ml of 0.15 M NaCl, 7 M urea, 0.05 M Tris-HCl, pH 7.0. The resulting CS/DS-oligosaccharides and core proteins (decorin) were removed by chromatography on a Q-Sepharose column (1.5 cm × 15 cm) as described above, except that the gradient started after 10 min of isocratic elution. The KSPGs were recovered after dialysis against water and lyophilization (yield 100 mg, 0.23% of the wet weight).

ELISA—Briefly, 1-μl portions of column fractions were diluted to 200 μl with coating buffer: Na2CO3 buffer, pH 9.6, containing 0.3% of sodium azide, and incubated at ambient temperature for 2 h in a multwell microtiter plate. The precoated wells were incubated with 200 μl of either antibody 5D4 (1:2000) or antibody LF95 (1:2500) for 1 h, followed by incubation for 1 h with 200 μl of enzyme-conjugated secondary antibodies: goat anti-mouse IgG peroxidase conjugate (1:1000) for 5D4 and goat anti-rabbit IgG peroxidase conjugate (1:2500) for LF95. The chromophore was developed by the addition of 200 μl of 3,3′,5,5′-tetramethylbenzidine (1 mg/10 ml in citrate buffer, pH 5.0, 20 μl hydrogen peroxide), and the absorbance read at 450 nm.

SDS-PAGE—Corneal samples were prepared as described below. Enzymatic deglycosylation with recombinant peptide N-glycosidase F was performed following the manufacturer’s instructions. Aliquots were removed after 1 h and 20 h of incubation time.

**FIG. 4.** SDS-PAGE of corneal KSPGs before and after various deglycosylations. Lane 1, standards; lane 2, endo-β-galactosidase digest of KSPGs; lane 3, keratanase digest of KSPGs; lane 4, keratanase II digest of KSPGs; lane 5, PNGase F digest of KSPGs (after 1 h of digestion); lane 6, PNGase F digest of KSPGs (after 20 h of digestion); lane 7, intact KSPGs. The band at 66 kDa in lane 2 is bovine serum albumin from the digestion buffer, and the band at 33 kDa in lanes 5 and 6 is recombinant PNGase F as indicated by the control (data not shown).

**FIG. 5.** Spherisorb S5 SAX ion-exchange chromatogram of keratanase II digest of corneal KSPGs. The column (1 cm × 25 cm) was eluted as described under “Experimental Procedures” and monitored on-line using conductivity and UV detection.
Enzymatic treatment with enzymes specific to keratan sulfate was carried out under the following conditions: 1 mg of KSPGs was dissolved in 100 μl of digestion buffer and incubated at 37°C with various enzymes. For endo-β-galactosidase (0.02 units), digestion was performed for 2 h in 0.05 M sodium acetate, pH 5.8, containing 0.2 mg of bovine serum albumin/ml. For keratanase II (0.01 units), digestion was carried out under the following conditions: 1 mg of KSPGs was dissolved in 100 μl of digestion buffer and incubated at 37°C for 20 h. The digest was then reduced with 4M NaBH₄ for 3 h and recovered after desalting on a Bio-Gel P2 column (300 × 7.8 mm).

Isolation of Oligosaccharides Derived by Keratanase II Digestion of KSPGs—Corneal KSPGs (100 mg) were dissolved in 5 ml of 10 mM sodium acetate, pH 6.5 and incubated with 0.25 units of keratanase II at 37°C for 30 h. The enzyme was inactivated by heating at 100°C for 5 min. The core proteins were removed by centrifugation at 100,000 g for 1 h. The digest was then reduced with 4 M NaBH₄ for 3 h and recovered after desalting on a Bio-Gel P2 column (1 cm × 10 cm).

The reduced keratanase II digest was chromatographed on a Sepharose CL-6B (83 cm × 1.5 cm) column and ion-exchange chromatography on a Mono-Q HR 10/10 column (data not shown).

The resulting KS was examined by 1H NMR spectroscopy (see below). Sugar compositional analysis using a CarboPac PA1 column after acid hydrolysis with 4 M trifluoroacetic acid (19) showed that the ratio of FucGlcNAcGalMan was 1.45:53:23. The weight-average molecular weight (Mₐ) of the KS chains was estimated (32) on a pre-calibrated Bio-Gel TSK-30 XL column (300 × 7.8 mm).

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**Corneal Keratan Sulfates**

**Preparation of Corneal Keratan Sulfate Chains—** Corneal KSPGs (40 mg) were dissolved in 0.8 ml of 1% SDS and boiled for 5 min. To this solution was added 7.2 ml of 0.1 M phosphate buffer, pH 7.3, containing 20 mM EDTA and 7% CHAPS, prior to the addition of 40 units of peptide N-glycosidase F. The enzyme was inactivated by heating at 100°C for 5 min after incubation at 37°C for 20 h. The digest was centrifuged at 100,000 × g for 1 h and the supernatant dialyzed against water and lyophilized. The released KS chains were further purified by gel-permeation chromatography on a Sepharose CL-6B (83 cm × 1.5 cm) column and ion-exchange chromatography on a Mono-Q HR 10/10 column (data not shown).

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eluate was monitored for the absorbance at 206 nm (Fig. 5). The fragments were recovered after desalting on a Bio-Gel P2 column.

**1H NMR Spectroscopy**

Samples were dissolved in 0.5 ml of 99.8% 2H₂O, buffered to pH 7.0 with phosphate, and referenced with internal sodium 3-trimethylsilyl[2,2,3,3-2H₄] propionate. After microfiltration through 0.45-μm nylon filters, samples were dried using a rotary concentrator and exchanged several times with 99.8% 2H₂O on once with 99.96% 2H₂O before final dissolution into 0.5 ml of 99.96% 2H₂O. 1H NMR spectra at 600 and 500 MHz were acquired using Bruker AMX600 and AM500 spectrometers, respectively. The 400-MHz 1H NMR spectra were obtained on a Jeol GSX400 spectrometer.

**RESULTS**

**KS Proteoglycans**—The entire population of KSPGs from the bovine corneal stroma (plus endothelium) were prepared after dissociative extraction and three stages of chromatography. The first step (Fig. 1) involved ion-exchange chromatography to separate the proteoglycans from protein contaminants. The material recovered (pooling bar) contained nearly all the KS, but substantial amounts of decorin (LF95-positive fractions) were present. The gel permeation chromatograph step (Fig. 2) permitted further purification on the basis of size. Finally, after chondroitin ABC lyase treatment to digest the glycosaminoglycans in the CS/DSPGs, the final ion-exchange step (Fig. 3) was used to separate the KSPGs from the core proteins of the CS/DSPGs. To establish the apparent number of proteoglycan species present in the preparation, the protein core sizes after several different enzymatic treatments were determined by SDS-PAGE (Fig. 4). Using the enzyme endo β-galactosidase, bands at 48, 44, and 36 kDa are obtained. These are similar to those reported by Funderburgh et al. (5) and seem to indicate the presence of at least three proteoglycan species, presumably those with the protein core sizes of 25 kDa, 37(A) kDa, and 40 kDa.

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**TABLE II**

| Residue | Protein | 1H* | 1H* | 1H* | 1H* | 1H* | 1H* |
|---------|---------|-----|-----|-----|-----|-----|-----|
| (A)     |         |     |     |     |     |     |     |
| SA2-3/6Gal-GlcNAc-Gal-GlcNAc-ol |         |     |     |     |     |     |     |
|         | E       | D   | C   | B   | A   |     |     |
| H2      | 1.726   | 1.622 | 1.813 | 1.833 |     |     |     |
| H3      | 2.709   | 2.778 | 2.798 | 2.772 |     |     |     |
| H4      |         |     |     |     |     |     |     |
| H5      |         |     |     |     |     |     |     |
| H6      |         |     |     |     |     |     |     |
| H7      |         |     |     |     |     |     |     |
| H8      |         |     |     |     |     |     |     |
| Gld     |         |     |     |     |     |     |     |
| H1      | 4.128   | 4.138 | 4.130 | 4.128 |     |     |     |
| H2      | 4.601   | 4.610 | 4.610 | 4.610 | 4.605 | 4.605 | 4.625 |
| H3      | 4.781   | 4.759 | 4.729 | 4.729 | 4.767 | 4.767 | 4.751 |
| H4      | 4.450   | 4.450 | 4.450 | 4.450 | 4.450 | 4.450 | 4.450 |
| H5      | 4.080   | 4.074 | 4.051 | 4.051 | 4.039 | 4.039 | 4.039 |
| H6      | 3.925   | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 |
| H7      | 3.919   | 3.847 | 3.847 | 3.847 | 3.847 | 3.847 | 3.847 |
| H8      | 4.019   | 4.019 | 4.019 | 4.019 | 4.019 | 4.019 | 4.019 |
| GlcNAc  | H1      | 4.769 | 4.741 | 4.735 | 4.715 | 4.718 | 4.715 | 4.715 |
| H2      | 3.831   | 3.875 | 3.875 | 3.875 | 3.875 | 3.875 | 3.875 |
| H3      | 3.781   | 3.781 | 3.781 | 3.781 | 3.781 | 3.781 | 3.781 |
| H4      | 3.781   | 3.781 | 3.781 | 3.781 | 3.781 | 3.781 | 3.781 |
| H5      | 4.041   | 4.041 | 4.041 | 4.041 | 4.041 | 4.041 | 4.041 |
| H6      | 3.970   | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 |
| H7      | 3.970   | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 |
| H8      | 3.970   | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 | 3.970 |

* from 1D spectrum

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**TABLE III**

| Residue | Protein | 6 | 20 | 17 |
|---------|---------|---|---|---|
| NeuAc   | H3<sub>6</sub> | 1.693* |     |     |
|         | H3<sub>5</sub> | 2.747* |     |     |
|         | H4      | 3.699 |     |     |
|         | H5      | 3.820 |     |     |
|         | H6      | 3.705 |     |     |
|         | H7      | 3.585 |     |     |
|         | H8      | 3.873 |     |     |
|         | H9      | 3.658 |     |     |
|         | H10     | 3.863 |     |     |
|         | NAc     | 2.045* |     |     |
| GlcNAc  | H1      | 4.744* | 4.680* |     |
|         | H2      | 3.781 | 3.975 |     |
|         | H3      | 3.595 | 3.773 |     |
|         | H4      | 3.552* | 4.007 |     |
|         | H5      | co<sub>3.66</sub> | 3.916 |     |
|         | H6      | co<sub>4.255</sub> | ca<sub>4.212</sub> |     |
|         | H7      | co<sub>4.328</sub> | ca<sub>4.212</sub> |     |
|         | NAc     | 2.052* | 2.022* |     |
| Gal or Gal(S) | H1 | 4.531 | 4.565 | 4.564 |
|         | H2      | 3.561 | 3.633 | 3.637 |
|         | H3      | 3.666 | 3.750 | 3.759 |
|         | H4      | 3.950 | 4.195 | 4.201 |
|         | H5      | co<sub>3.93</sub> | ca<sub>3.945</sub> |     |
|         | H6      | 3.665 | 4.198 | 4.204 |
|         | H7      | co<sub>3.92</sub> | 4.235 | 4.237 |
|         | H8      | 4.232 | 4.241 | 4.217 |
|         | H9      | 3.353 | 4.335 | 4.340 |
|         | NAc     | 2.071* | 2.070* | 2.071* |

* from 1D spectrum

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**Fig. 7. 600-MHz COSY-45 spectrum of oligosaccharide alditol (19) at 50 °C. NeuAcα2–3Gal(S)β1–4GlcNAc(S)β1–3Galβ1–4GlcNAc(S)-ol is shown.**
37(B) kDa. The comparative core protein sizes after enzyme treatment increase in the order: recombinant peptide N-glycosidase F, endo β-galactosidase, keratanase, and then keratanase II, reflecting that the relative proximity of the enzyme cleavage sites to the core protein is in this sequence. The pattern of core proteins released by recombinant peptide N-glycosidase F (EC 3.2.2.18) in this study is slightly different from that observed by Funderburgh et al. (5), who used N-glycanase (EC 3.5.1.52), and this, perhaps, is due to different fine specificities in the enzymes used.

**KS Chains**—The KS chains prepared from these KS PGs by peptide N-glycosidase F digestion were assessed for their molecular sizes on a calibrated TSK-30 column (32). From these data (not shown), it is clear that the total KS population exhibits considerable chain size polydispersity ($M_n$ 5000–30,000).

**Keratanase II-derived Fragments**—The oligosaccharides were derived from a limit digest of the KS proteoglycan population and thus lack the linkage regions, which remained attached to the protein cores. After borohydride reduction, these fragments were chromatographed on a Spherisorb S5 SAX column (Fig. 5) and recovered. The resulting oligosaccharides listed in Table I can be categorized into those deriving from either the repeat regions or the capping regions.

**Oligosaccharides Containing Repeating Disaccharide Units**—The oligosaccharides $1, 8, 9, 14, 16,$ and $22$ have 1H NMR spectra identical with those of oligosaccharides, $R_1–R_6$, derived from cartilage keratan sulfate (33, 34) and have the structures shown in Table I. The one-dimensional spectrum for $19$ (Fig. 6) resembles those from oligosaccharides $C_1–C_5$. The spectrum may be fully assigned with the aid of COSY-45 data (Fig. 7). Complete sets of resonances corresponding to five sugar residues are found (see Table II). Signals at 4.22 and 4.26 ppm connect through to a series of protons with positions characteristic for α(2–3)-linked N-acetylgalactosaminic acid, residue E. The presence of an internal 6-sulfated N-acetylglucosamine (residue C) is indicated by the anomeric proton signal at 4.73 ppm through to the non-equivalent methylene resonances at 4.293 and 4.445 ppm (Fig. 7). A reducing terminal 6-sulfated N-acetylgalactosaminol, residue A, shows connections almost identical to those found for oligosaccharide $C_2$ (see above), in which GlcNAc(S)-ol is attached to an unsulfated galactose (33). The two remaining sets of signals can be assigned to galactose residues B and D by reference to previously presented data (33, 35).
structure of 19 is thus NeuAc\(\alpha\)2–3Gal\(\beta\)1–4GlcNAc\(\beta\)1–3Gal\(\beta\)1–4GlcNAc(S)-ol.

Oligosaccharides Containing \(\alpha(2\rightarrow6)\)-linked N-Acetyleneuraminic Acid—The oligosaccharides 11 and 21 have \(^1\)H NMR spectra (data not shown) identical to those for the previously characterized C1 and C3 from KS type II (33, 34). Therefore, the structures of 11 and 21 are as follows: 11, NeuAc\(\alpha\)2–6Gal\(\beta\)1–4GlcNAc\(\beta\)1–3Gal\(\beta\)1–4GlcNAc(S)-ol; 21, NeuAc\(\alpha\)2–6Gal\(\beta\)1–4GlcNAc\(\beta\)1–3Gal\(\beta\)1–4GlcNAc(S)-ol.

The \(^1\)H NMR spectrum of 6 (data not shown) also displays signals characteristic of an \(\alpha(2\rightarrow6)\)-linked N-acetyleneuraminic acid residue with H-3\(_{ax}\) at 1.693 ppm. However, a signal at 2.747 ppm has not, hitherto, been observed from an \(\alpha(2\rightarrow6)\)-linkage environment. The spectrum is simple, and the complete assignment of the chemical shifts (see Table III) for 6 is consistent with the following trisaccharide structure: NeuAc\(\alpha\)2–6Gal\(\beta\)1–4GlcNAc(S)-ol.

Oligosaccharides Containing \(\alpha(2\rightarrow3)\)-linked N-Glycolyneuraminic Acid—The \(^1\)H NMR spectra of 18 and 23 (data not shown) show structural similarity. Both display signals at about 1.83, 2.77, and 4.128 ppm, characteristic of H-3\(_{ax}\), H-3\(_{eq}\), and N-glycolyl methylene protons, respectively, in N-glycolyneuraminic acid \(\alpha(2\rightarrow3)\)-linked to galactose (36). In addition, both spectra show three anomic proton signals (4.55–4.8 ppm) and two N-acetyl methyl proton signals in the range 2.0–2.1 ppm. These features suggest that 18 and 23 are pen-
tasaccharides with the core structure, NeuGcα2–3Galβ1–4GlCNacβ1–3Galβ1–4GlCNac(S)-ol is shown.

The spectrum for fraction 10 (Fig. 8) shows two groups of signals with intensity ratio 2:1, representing two components: 10i and 10ii. The spectral profile of 10ii is similar to those of 18 and 23; this includes signals typical for an α(2–3)-linked N-glycolyleneuraminic acid residue. It may, therefore, possess the same core structure as 18 and 23.

The chargedensity of each oligosaccharide may be estimated from the elution position on the ion-exchange column. 10ii elutes in the region between di- and trisulfated repeat-unit oligosaccharides. It elutes close to 11, a sialylated, disulfated pentasaccharide. 18 elutes between trisulfated and tetrasulfated repeat-unit oligosaccharides in the group of sialylated, trisulfated pentasaccharides such as 19 and 21. Oligosaccharide 23 carries a higher chargedensity, eluting after the tetrasulfated tetrasaccharide, 22, in the region of the sialylated, tetrasulfated pentasaccharide C5. Thus 10ii, 18 and 23 contain two, three and four sulfate groups, respectively. The structure of 23 is: NeuGcα2–3Galβ1–4GlCNacβ1–3Galβ1–4GlCNac(S)-ol.

The spectrum of 10i shows an anomeric proton signal at 4.741 ppm (Fig. 8) and an N-acetyl methyl signal at 2.045 ppm (data not shown), indicating the presence of an internal 6-sulfated N-acetylgalactosamine (37). The other sulfate group should be on the reducing terminal N-acetylgalactosaminic acid residue since keratanase II requires a sulfated N-acetylgalactosaminyl residue. It may, therefore, possess the same core structure as NeuGcα2–3Galβ1–4GlCNacβ1–3Galβ1–4GlCNac(S)-ol.

The spectrum of 10ii shows signals at 1.726, 2.709, and 4.128 ppm, which are characteristic of H-3α, H-3eq, and N-glycolyleneuraminic acid respectively, of an N-glycolyleneuraminic acid α(2–6)-linked to a galactose (36). The structure of 10ii is NeuGcα2–6Galβ1–4GlCNac(S)β1–3Galβ1–4GlCNac(S)-ol.

**TABLE IV**

| Residue | Proton | 7 | 15 | R2* | R5* |
|---------|--------|---|----|-----|-----|
| Galα    | H1     | 5.152* | 5.152* |       |     |
| (E)     | H2     | 3.876  | 3.875* |       |     |
|         | H3     | 3.960  | 3.961 |       |     |
|         | H4     | 4.041* | 4.041* |       |     |
|         | H5     | 4.197  |       |       |     |
|         | H6     | ca. 3.754 | ca. 3.75 |       |     |
| Gal     | H1     | 4.597* | 4.614* | 4.535 | 4.553 |
| (D)     | H2     | 3.675  | 3.672* | 3.553 | 3.550 |
|         | H3     | 3.790  | 3.794 | 3.686 | 3.686 |
|         | H4     | 4.182  |       | 3.949 | 3.948 |
|         | H5     | ca. 3.745 | ca. 3.765 |       |     |
|         | H6     | -      | -    | -    |     |
| GlcNAc  | H1     | 4.755* | 4.775* | 4.756 | 4.774 |
| (B)     | H2     | 3.842  | 3.836 | 3.834 | 3.827 |
|         | H3     | 3.777  | 3.776 |       | 3.746 |
| Gal     | H1     | 4.560* | 4.563* | 4.560 | 4.563 |
|         | H2     | 3.626  | 3.640* | 3.626 | 3.641 |
|         | H3     | 3.728  | 3.756 | 3.727 | 3.754 |
|         | H4     | ca. 4.205 | ca. 4.174 | 4.179 | 4.197 |
|         | H5     | -      | 3.936 | -    | 3.925 |
|         | H6     | -      | 4.205 | -    | 4.192 |
|         | H6’    | 4.241  |       | -    | 4.237 |
| GlcNAc-ol| H1    | 3.683  | 3.753 | 3.685 | 3.744 |
| (A)     | H1’    | 3.772  | 3.788 | 3.773 | 3.788 |
|         | H2     | 4.276  | ca. 4.22 | 4.272 | ca. 4.21 |
|         | H3     | 3.977  | 3.999* | 3.978 | 3.999 |
|         | H4     | 3.933  | 3.921 | 3.932 | 3.918 |
|         | H5     | 4.140  | 4.149* | 4.140 | 4.149 |
|         | H6     | 4.255  | 4.223 | 4.252 | 4.215 |
|         | H6’    | 4.332  | 4.337 | 4.330 | 4.332 |
| NAc     | 2.059* | 2.070* | 2.059 | 2.070 |     |

* from 1D spectrum a R2 and R3 data are from Brown et al. (33,34)

**Oligosaccharides Containing Terminal α(1–3)-linked Galactose**—Oligosaccharides 7 and 15 have similar one-dimensional 1H NMR spectra (Fig. 10). Both contain three anomeric proton signals in the range 4.5–4.8 ppm, and a fourth near 5.15 ppm. There is also a cluster of resonances at 4.14–4.15 ppm, attributable to H-5 of a reducing terminal 6-sulfated N-acetylgalactosaminol (33, 34). Thus, the two fragments appear to be pentasaccharides. These spectra have been almost completely assigned with the aid of two-dimensional COSY-45 data; a correlation spectrum for 15 is shown in Fig. 11.

For both 7 and 15, sets of connected resonances corresponding to five sugar residues have been observed; data are summarized in Table IV. One set is unique in that the anomeric proton resonance falls well downfield, at 5.152 ppm, with a small coupling constant to H-2 (3.95 Hz). This indicates the presence of an α-linkage. For 7, this anomeric proton signal connects via H-2 at 3.876 ppm, H-5 at 3.960 ppm, and H-4 at 4.041 ppm to H-5 at 4.197 ppm and finally to H-6, at −3.754 ppm. The shift positions of H-1 and H-5, in combination with small coupling constants J1,2, J3,4, and J4,5, are characteristic for a non-reducing terminal α(1–3)-linked galactose (39).

The other four residues are identified by comparison with data for the R2 (Galβ1–4GlCNacS)β1–3Galβ1–4GlCNac(S)-ol (33, 34). These results show that the structure of 7 is Galα1–3Galβ1–4GlCNac(S)β1–3Galβ1–4GlCNac(S)-ol.
The COSY-45 spectrum (Fig. 11) for 15 can be interpreted in a similar manner. The \(a(1–3)\)-linked residue resonances are at virtually identical shift positions to those in 7. The other four signal sets are assigned by comparison with data from R5 (Gal\(\beta1–4\)GlcNAc(S)\(\beta1–3\)Gal(S)\(\beta1–4\)GlcNAc(S)-ol) (33, 34). Thus 15 has the following structure: Gal\(a1–3\)Gal\(\beta1–4\)GlcNAc(S)\(\beta1–3\)Gal(S)\(\beta1–4\)GlcNAc(S)-ol.

Oligosaccharides Capped with N-Acetylated Residues—Oligosaccharide 20 elutes in the region between trisulfated and tetrasulfated repeat-unit tetrasaccharides. In this region only sialylated, trisulfated pentasaccharides have been observed in studies of cartilage KS (33, 34). However, the \(^1\)H NMR spectrum for 20 shows no sialic acid resonances (Fig. 12a). It therefore must be a new oligosaccharide derived from corneal KS.

The one-dimensional (Fig. 12a) and two-dimensional \(^1\)H NMR spectra for 20 demonstrate sets of chemical shifts corresponding to three sugar residues, with assignments summarized in Table III. Residues A and B are clearly sulfated N-acetylgalactosaminitol and internal sulfated galactose, respectively (33, 34). Residue C has an anomeric proton signal at 4.744 ppm, which connects to H-2 at 3.781 ppm, thence to H-3 at 3.595 ppm, and H-4 at 3.552 ppm. Although connection between H-4 and H-5 is lost, there is a resonance for H-5 at \(-3.66 \) ppm connecting to a non-equivalent methylene pair at \(-4.255 \) and \(-4.328 \) ppm. These shifts are almost identical to those reported for the non-reducing terminal 6-sulfated N-acetylgalactosamine in oligosaccharide KS4R (GlcNAc(S)\(\beta1–3\)Gal(S)\(\beta1–4\)GlcNAc(S)-ol) (40). The structure of 20 is, therefore: GlcNAc(S)\(\beta1–3\)Gal(S)\(\beta1–4\)GlcNAc(S)-ol.

Oligosaccharide 17 elutes just after the trisulfated repeat-unit tetrasaccharides. The one-dimensional (Fig. 12b) and two-dimensional \(^1\)H NMR (Fig. 13) spectra demonstrate that it is a trisaccharide. Responses corresponding to GlcNAc(S)-ol, residue A, and Gal(S), residue B, are located at positions almost identical with those for the corresponding signals from 20. The third set of connected resonances is, however, quite distinct. The anomeric proton signal at 4.680 ppm has a novel shift position. It connects to H2 at 3.973 ppm with a coupling constant of 8.34 Hz, suggesting that it has a \(\beta\)-configuration. Connections continue to 3.773 ppm (H-3) and 4.007 ppm (H-4). While the connection between H-4 and H-5 is weak, since \(J_{4,5} \approx 1 \) Hz, those between H-5 (at 3.916 ppm) and H6,6' (both at \(-4.212 \) ppm) are clearly visible on the COSY-45 spectrum (Fig. 13). These shift values and coupling constants are similar to those reported for a non-reducing terminal 6-sulfated galactose.
(33, 34), except that H-1 and H-2 are shifted significantly downfield. The one-dimensional spectrum also shows an extra N-acetyl methyl proton signal at 2.052 ppm, together with a signal at 2.071 ppm arising from the N-acetylglucosaminol. These data (summarized in Table III) suggest that this sugar must be a β(1–3)-linked 6-sulfated N-acetylgalactosamine (41). The structure of 17 is, therefore, GalNAc(S)β1–3Gal(S)β1–4GlcnAc(S)-ol.

**NMR Spectrum of Peptide N-Glycosidase F Released and Reduced KS Chains**—A partial 600-MHz 1H NMR spectrum for the peptide N-glycosidase F released and borohydride reduced KS chains is shown in Fig. 14. This shows several interesting features. First, the absence of a complex GlcNAc anomeric signal at 5.05 ppm indicates that the GlcNAc-Asn bond has been cleaved by the peptide N-glycosidase F (42). Second, several anomeric signals in the range of 4.60–5.20 ppm can now be assigned. The anomeric proton of the α(1–3)-galactose-capping residues resonates at 5.15 ppm (see above). The complex signals at 5.13 ppm and 4.90–4.95 ppm correspond to the H-1 of the α(1–3)-mannose and α(1–6)-mannose residues in the linkage region. The doublet at 4.905 ppm corresponds to H-1 of α(1–6)-linked fucose attached to GlcNAc in the linkage region (42). The small doublet in the range 4.77–4.79 ppm (marked *) corresponds to H-1 of GlcNAc in the capping sequence, NeuAcα(2–6)-Gal-GlcNAc(S)-Gal (23). The large resonance at 4.7–4.76 ppm is clearly the H-1 of GlcNAc. The small doublet at 4.665 ppm corresponds to the H-1 of capping GalNAc(S).

The sets of resonances at 1.71 and 2.69 ppm are typical of α(2–6)-linked sialic acids (both NeuAc and NeuGc) and those at 1.81 and 2.76 ppm of α(2–3)-linked sialic acids. It is clear that this corneal sample contains more α(2–6)- than α(2–3)-linked sialic acids unlike skeletal KS. The major methyl resonance of fucose is seen at 1.235 ppm corresponding to α(1–6)-linked fucose in the linkage region (42), but a smaller signal at 1.164 ppm can be assigned to α(1–3)-linked fucose (43) from the repeat regions (see below).

**DISCUSSION**

In this study seven families of chain caps have been isolated from the entire corneal KS population and their proportions are shown in Fig. 15. The most surprising aspect of this research is the diversity of these chain-capping structures. Thus, the terminal residues and their linkages, NeuAcα2–3, NeuAcα2–6, NeuGcα2–3, NeuGcα2–6, Galα1–3, GlcNAc(S)β1–3, and GalNAc(S)β1–3, have all been identified in this bovine corneal KS PG preparation. In principle, Galβ1–4 is a potential chain-capping residue, but separate studies (data not shown) using the enzyme keratanase indicate that Galβ1–4 is unlikely to be a chain terminator.

Only two of these caps, NeuAcα2–3 and NeuAcα2–6, occur in either skeletal KS or in fibromodulin-derived N-linked KS from bovine articular cartilage. Thus, the other five, NeuGcα2–3, NeuGcα2–6, Galα1–3, GlcNAc(S)β1–3, and GalNAc(S)β1–3, have tissue-specific distribution in bovine cornea but not cartilage. The caps, NeuGcα2–3 and NeuGcα2–6, would not be expected to occur in normal adult human tissues (44) and are...
highly antigenic in humans. The Galα1–3Galβ1–4GlcNAc structure is also not observed in humans because of a non-functional α(1–3)-galactosyltransferase (45). It seems possible that the diversity of chain caps is related to the presence of three or more KSPGs in cornea, but an assessment of probable cap function will require identification of the antenna and PG distribution of individual caps.

The fucose content of the corneal KS was addressed in three ways. First, the carbohydrate analysis of the released KS chains showed 1.45 fucose per triple-mannose. As discussed below, this is an overestimate of fucose (perhaps by 30%) because of the underestimation of mannose. Second, the 1H NMR spectrum (Fig. 14) of the released KS chains shows a 10:1 ratio of α(1–6)- to α(1–3)-fucose, and as a KS chain has maximally one α(1–6)-fucose in its linkage region (such oligosaccharides have not been studied here), it is clear that the α(1–3)-fucose content cannot be higher than one per 10 chains. Third, in this study two fucose-containing oligosaccharides, Galβ1–4GlcNAc(S)β1–3Galβ1–4(Fucα1–3)GlcNAc(S)-ol and Galβ1–4(Fucα1–3)GlcNAc(S)β1–3Galβ1–4GlcNAc(S)-ol (33, 34), have been identified (data not shown) as contaminants in oligosaccharides 6 and 7, respectively. The small amount of α(1–3)-fucose is distributed among several fragments (33, 34) and is, therefore, difficult to study by keratanase II analysis, but it is likely to be found in the number-average molecular weight of about 1,800. If the two estimated M<sub>r</sub> values of 10,000 and 17,000 are used for the KS chains, then the remaining repeat and capping regions would be expected to have masses of between 8200 and 15200. Dividing these by 5500 yields between 1.5 and 2.7 caps (cleavable by keratanase II) per average KS chain. This is broadly consistent with a bi-antennary model (25, 26). However, it must be emphasized that KS chains from different corneal KSPGs may have discrete structures, and thus no single generalized model of corneal KS may be appropriate.

This study has characterized a large number of, hitherto, unrecognized capping structures in corneal KS. However, further studies will be required to identify how the caps are distributed between the three known KSPGs and their antennae, and whether their relative contents vary with age and animal species. It is clearly a priority to purify the individual KSPGs and characterize their keratan sulfates either by spectroscopic or fingerprinting methods (48).

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REFERENCES
1. Berman, E. R. (1991) in Biochemistry of the Eye (Blakemore, C., ed) pp. 89–150, Plenum Publishing Corp., New York
2. Scott, J. E. and Haigh, M. J. (1990) J. Biol. Chem. 265, 1052–1059
3. Scott, J. E. (1992) J. Biol. Biochem. Biophys. 296, 190–197
4. Li, W., Vergnes, J. P., Cornuet, P. K., and Hassell, J. R. (1992) Arch. Biochem. Biophys. 296, 190–197
5. Funderburgh, J. L., and Conrad, G. W. (1990) J. Biol. Chem. 265, 8297–8303
6. Funderburgh, J. L., Funderburgh, M. L., Mann, M. M., and Conrad, G. W. (1991) J. Biol. Chem. 266, 14226–14231
7. Blechberger, T. C., Vergnes, J. P., Hempel, J., and Hassell, J. R. (1992) J. Biol. Chem. 267, 347–352
8. Funderburgh, J. L., Funderburgh, M. L., Brown, S. J., Vergnes, J. P., Hassell, J. R., Mann, M. M., and Conrad, G. W. (1995) J. Biol. Chem. 269, 11874–11880
9. Corpuz, L. M., Funderburgh, J. L., Funderburgh, M. L., Bottomley, G. S., Prakash, S., and Conrad, G. W. (1996) J. Biol. Chem. 271, 7539–7543
10. Meyer, K., Linker, A., Davidson, E. A., and Weissman, B. (1983) J. Biol. Chem. 258, 611–616
11. Meyer, K. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (Balazs, E. A., ed) pp. 15, Academic Press, New York
12. Nieduszynski, I. A., Huckerby, T. N., Dickenson, J. M., Brown, G. M., Tai, G. H., Morris, H. G., and Eady, S. (1990) J. Biol. Chem. 265, 243, 245–245
13. Kruszynska, T., Reinhold, V. N., Margolis, R. K., and Margolis, R. U. (1987) Biochem. J. 245, 229–234
14. Bray, A., Lieberman, R., and Meyer, K. (1967) J. Biol. Chem. 242, 3737–3740
15. Hopwood, J. J., and Robinson, C. H. (1974) Biochem. J. 141, 517–526
16. Dickens, J. M., Huckerby, T. N., and Nieduszynski, I. A. (1990) Biochem. J. 269, 55–59
17. Bhavanandan, V. P., and Meyer, K. (1968) J. Biol. Chem. 243, 1052–1059
18. Thorson, D. J., Morris, H. G., Cockin, G. H., Huckerby, T. N., Nieduszynski, I. A., Carlestedt, I., Hardingham, T. E., and Ratcliffe, A. (1989) Biochem. J. 260, 177–282
19. Tai, G. H., Brown, G. M., Morris, H. G., Huckerby, T. N., and Nieduszynski, I.
A. (1991) Biochem. J. 273, 307–310
20. Brown, G. M., Huckerby, T. N., Morris, H. G., and Nieduszynski, I. A. (1992) Biochem. J. 286, 235–241
21. Toda, N., and Seno, N. (1970) Biochim. Biophys. Acta 208, 227–235
22. Dickenson, J. M., Huckerby, T. N., and Nieduszynski, I. A. (1991) Biochem. J. 278, 779–785
23. Dickenson, J. M., Huckerby, T. N., and Nieduszynski, I. A. (1992) Biochem. J. 282, 267–273
24. Tai, G. H., Morris, H. G., Brown, G. M., Huckerby, T. N., and Nieduszynski, I. A. (1992) Biochem. J. 286, 231–234
25. Nilsson, B., Nakazawa, K., Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1983) J. Biol. Chem. 258, 6056–6063
26. Oeben, M., Keller, R., Stuhlsatz, H. W., and Greiling, H. (1987) Biochem. J. 248, 85–93
27. Houssell, E. F., Feeney, J., Scudder, P., Tang, P. W., and Feizi, T. (1986) Eur. J. Biochem. 157, 375–384
28. Seno, N., Meyer, K., Anderson, B., and Hoffman, P. (1985) J. Biol. Chem. 240, 1095–1010
29. Choi, H. U., and Meyer, K. (1975) Biochem. J. 151, 543–553
30. Farndale, R. W., Sayers, C. A., and Barrett, A. J. (1982) Connect. Tissue Res. 9, 247–248
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Dickenson, J. M., Morris, H. G., Nieduszynski, I. A., and Huckerby, T. N. (1990) Anal. Biochem. 180, 271–275
33. Brown, G. M., Huckerby, T. N., and Nieduszynski, I. A. (1994) Eur. J. Biochem. 224, 281–308
34. Brown, G. M., Huckerby, T. N., Morris, H. G., Abram, B. L., and Nieduszynski, I. A. (1994) Biochemistry 33, 4836–4846
35. Huckerby, T. N., Dickenson, J. M., and Nieduszynski, I. A. (1992) Magn. Reson. Chem. 30, S134–S141
36. Savage, A. V., Koppen, P. L., Schiphorst, W. E. C. M., Trippelvitz, L. A. W., van Halbeek, H., Vliegenthart, J. F. G., and Vandeneijnden, D. H. (1986) Eur. J. Biochem. 160, 123–129
37. Huckerby, T. N., Dickenson, J. M., and Nieduszynski, I. A. (1990) Magn. Reson. Chem. 28, 786–791
38. Nakazawa, K., Ito, M., Yamagata, T., and Suzuki, S. (1989) in Keratan Sulfate: Chemistry, Biology and Chemical Pathology (Greiling, H., and Scott, J. E., eds) pp. 99–110, The Biochemical Society, London
39. Dorland, L., van Halbeek, H., and Vliegenthart, J. F. G. (1984) Biochim. Biophys. Res. Commun. 122, 859–866
40. Huckerby, T. N., Dickenson, J. M., Tai, G. H., Lauder, R. M., Brown, G. M., and Nieduszynski, I. A. (1993) Magn. Reson. Chem. 31, 394–398
41. Welti, D., Rees, D. A., and Welsh, E. J. (1979) Eur. J. Biochem. 94, 505–514
42. Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. 41, 269–374
43. Huckerby, T. N., Nieduszynski, I. A., Brown, G. M., and Cockin, G. H. (1991) Glycoconjugate J. 8, 39–44
44. Varki, A. (1992) Glycoconjugate J. 8, 25–40
45. Galili, U. (1993) Immunol. Today 14, 480–482
46. Kannagi, R., Fukuda, M. N., and Hakomori, S. (1982) J. Biol. Chem. 257, 4438–4442
47. Hardy, M. R., Townsend, R. R., and Lee, Y. C. (1988) Anal. Biochem. 170, 54–62
48. Brown, G. M., Nieduszynski, I. A., Morris, H. G., Abram, B. L., Huckerby, T. N., and Block, J. A. (1995) Glycobiology 5, 311–317
