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Structural studies on sialylated and sulphated *O*-glycosidic mannose-linked oligosaccharides in the chondroitin sulphate proteoglycan of brain

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We have previously described the structures of neutral and sialylated *O*-glycosidic mannose-linked tetrasaccharides and keratan sulphate polysaccharide chains in the chondroitin sulphate proteoglycan of brain. The present paper provides information on a series of related sialylated and/or sulphated tri- to penta-saccharides released by alkaline-borohydride treatment of the proteoglycan glycopeptides. The oligosaccharides were fractionated by ion-exchange chromatography and gel filtration, and their structural properties were studied by methylation analysis and fast-atom-bombardment mass spectrometry. Five fractions containing [^{35}S]sulphate-labelled oligosaccharides were obtained by ion-exchange chromatography, each of which was eluted from Sephadex G-50 as two well-separated peaks. The apparent M_r values of both the large- and small-molecular-size fractions increased with increasing acidity (and sulphate labelling) of the oligosaccharides. The larger-molecular-size fractions contained short mannose-linked keratan sulphate chains of M_r 3000–4500, together with some asparagine-linked oligosaccharides. The smaller tri- to penta-saccharides, of M_r 800–1400, appear to have a common GlcNAc(β1–3)Manol core, and to contain one to two residues of sialic acid and/or sulphate.

INTRODUCTION

The chondroitin sulphate proteoglycan of brain has an M_r of approx. 300000 and is present in the extracellular space of early postnatal brain, after which period it progressively assumes an intracellular (cytoplasmic) localization in neurons and astrocytes (Aquino *et al.*, 1984*a*,*b*). Most of the proteoglycan is readily soluble in a phosphate-buffered-saline extract of brain, where it accounts for less than 1% of the soluble brain protein (Kiang *et al.*, 1981). The proteoglycan is composed of 56% protein, 24% glycosaminoglycans (predominantly chondroitin 4-sulphate) and 20% glycoprotein oligosaccharides, which are of both *N*- and *O*-glycosidic types (Kiang *et al.*, 1981; Klinger *et al.*, 1985; Krusius *et al.*, 1986).

In addition to galactosyl-(β1→3)-*N*-acetylgalactosamine and its mono- and di-sialyl derivatives, we have previously reported the presence in the chondroitin sulphate proteoglycan of a series of novel mannose-linked *O*-glycosidic oligosaccharides that can be isolated by mild alkaline-borohydride treatment of the proteoglycan glycopeptides, and have the sequence GlcNAc(β1–3)-Manol at their proximal ends (Finne *et al.*, 1979; Krusius *et al.*, 1986). These include GlcNAc(β1–3)Manol itself, Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Manol, Neu-Ac(α2–3)Gal(β1–4)GlcNAc(β1–3)Manol and mannose-linked keratan sulphate chains composed of disaccharide repeating units consisting of Gal(β1–4)GlcNAc-6-*O*-SO_4(β1–3). Amino acid and carbohydrate analyses before and after alkaline-borohydride treatment demonstrated that over half of the carbohydrate–peptide linkages in the proteoglycan are of the mannosyl-*O*-serine/threonine type (Krusius *et al.*, 1986). In the present paper we provide information about the structural properties of other mannose-linked *O*-glycosidic oligosaccharides, which, because of their lower concentrations and the presence of a large number of related oligosaccharides with similar physicochemical properties, could not be as thoroughly characterized in our previous studies.

EXPERIMENTAL

Fractionation of glycopeptides and oligosaccharides

The chondroitin sulphate proteoglycan was isolated from a phosphate-buffered-saline extract of rat brain by chromatography on DEAE-cellulose and Sepharose CL-6B as described previously (Kiang *et al.*, 1981). Labelled oligosaccharides were prepared from proteoglycan isolated from 30-day-old rats after bilateral intracerebral injection of 5–7 μl of 0.9% NaCl containing carrier-free $Na_2{}^{35}SO_4$ (50 mCi/ml) 18 h before decapitation. Glycopeptides were prepared by Pronase digestion, and, after precipitation of the glycosaminoglycans by cetylpyridinium chloride, the glycopeptides were purified by gel filtration on Sephadex G-25 (fine grade) (Kiang *et al.*, 1981).

Alkali-labile oligosaccharides were liberated by treatment of the glycopeptides (corresponding to 1 mg of

Abbreviations used: Manol (in sequences), mannitol; f.a.b., fast atom bombardment.

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proteoglycan protein/ml of base) with 1 M-$NaBH_4$ (or, in certain cases, NaB^2H_4) in 0.05 M-NaOH for 16 h at 45 °C (Carlson, 1968). After incubation, excess $NaBH_4$ was destroyed with acetic acid, the sample was passed through a column of Dowex-50 X4 (H^+ form) to remove Na^+ ions, and boric acid was removed by repeated evaporations with HCl/methanol (1:1000, v/v). Alkali-labile oligosaccharides were fractionated on DEAE-Sephadex A-25 by elution with a linear gradient of pyridine/acetate buffer, pH 5.0 (Finne, 1975). The oligosaccharides were further fractionated by gel filtration on Sephadex G-50 as described in the legend to Fig. 2.

Analytical methods

Monosaccharides were determined after methanolysis as their trimethylsilyl derivatives by g.l.c. on a column of 2.2% OV-101. Hexitols were also analysed after hydrolysis in 2 M-HCl for 2 h at 100 °C as their peracetylated derivatives (Kim *et al.*, 1967) by g.l.c. on a column of 1% OV-225 and m.s. The peracetylated hexitols were detected by total ion current and mass fragmentography at *m/z* 217 (Kärkkäinen, 1969). Iditol was used as an internal standard. Oligosaccharides were methylated by the method of Hakomori (1964) with the methylation reagent prepared from potassium t-butoxide (Finne *et al.*, 1980). The methylated oligosaccharides were degraded by acetolysis and acid hydrolysis (Stellner *et al.*, 1973), and the partially methylated alditol acetates were detected by total ion current and mass fragmentography as described previously (Rauvala *et al.*, 1981). The alkali-labile oligosaccharides were analysed after hydrolysis of their sialic acid residues (0.1 M-HCl, 1 h, 80 °C) as their trimethylsilyl derivatives by g.l.c. and m.s. as described previously (Finne *et al.* 1977).

Samples examined by f.a.b. m.s. were run on a VG ZAB-SE mass spectrometer (VG Analytical, Manchester, U.K.), with triethanolamine as the liquid matrix.

Other methods

Protein content of the isolated proteoglycan was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Sialic acid in the column effluents was determined colorimetrically. Uronic acid was determined by a modified carbazole reaction (Mathews & Cifonelli, 1965) with absorbance measured at both 530 and 430 nm. The amount of uronic acid was calculated on the basis of the difference of the absorbance values.

For endo-β-galactosidase treatment of labelled glycopeptides, samples containing less than 100 μg of oligosaccharide were digested for 16 h at 37 °C under toluene in 0.1 ml of 50 mM-sodium acetate buffer, pH 6.0, containing 0.1 mg of endo-β-galactosidase from *Escherichia freundii* (Nakagawa *et al.*, 1980), which was kindly provided by Dr. Y.-T. Li (Tulane University School of Medicine).

The molecular size of the chondroitin sulphate proteoglycan was determined by gel filtration on a Sepharose CL-2B column (0.9 cm × 65 cm) eluted with 50 mM-sodium acetate buffer, pH 5.8, containing 4 M-guanidinium chloride, by using the calibration relationships for cartilage chondroitin sulphate proteoglycan subunits described by Ohno *et al.* (1986). The void volume was determined with Blue Dextran and the total column volume by the elution peak of $Na_2{}^{35}SO_4$ or acetone (which gave the same values).

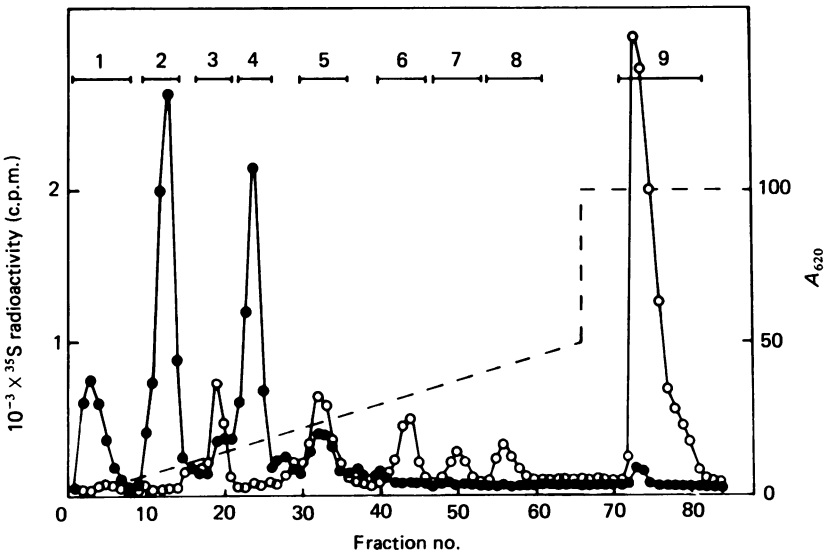

Fig. 1. Ion-exchange chromatography of oligosaccharides released by NaOH/$NaBH_4$

The sample was dissolved in 10 ml of 5 mM-pyridine/acetate buffer, pH 5.0, and applied to a DEAE-Sephadex A-25 column (1 cm × 25 cm) equilibrated with the same buffer. The column was first eluted with 5 ml of 10 mM buffer and then with 15 ml of 50 mM-pyridine/acetate buffer, pH 5.0. Elution was continued with a linear gradient of 50–1250 mM-pyridine/acetate buffer, pH 5.0, in 400 ml (broken line). At the end of the gradient, the column was eluted with 2 M-pyridine/acetate buffer, pH 5.0. Fractions (5.5 ml) were collected, and samples were analysed for sialic acid (A_{620}, ●) and ^{35}S radioactivity (○). Fractions indicated by the bars were pooled.

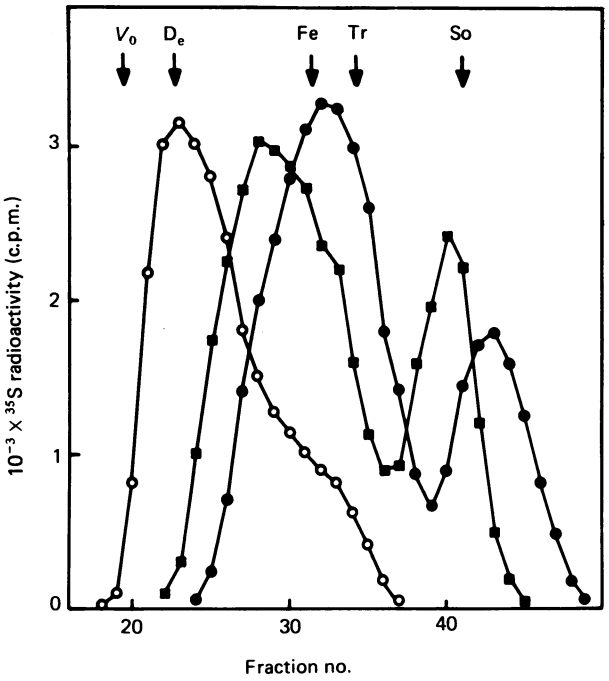

Fig. 2. Gel filtration on Sephadex G-50 (fine grade) of ^{35}S-labelled oligosaccharides isolated by anion-exchange chromatography

The column (2 cm × 75 cm) was eluted with 0.1 M-pyridine/acetate buffer, pH 5.0, and 4.5 ml fractions were collected. Samples of the effluent were monitored for ^{35}S radioactivity: ●, fraction 7; ■, fraction 8; ○, fraction 9. For each DEAE-Sephadex fraction, the tubes from the subsequent Sephadex G-50 elution representing peaks of large- or small-molecular-size oligosaccharides were pooled and designated I and II respectively. The void volume (V_0) and the elution volumes of Dextran T10 (D_e), triantennary complex-type glycopeptides from fetuin (Fe), biantennary complex-type glycopeptides from transferrin (Tr) and NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAcol (So) are indicated for comparison.

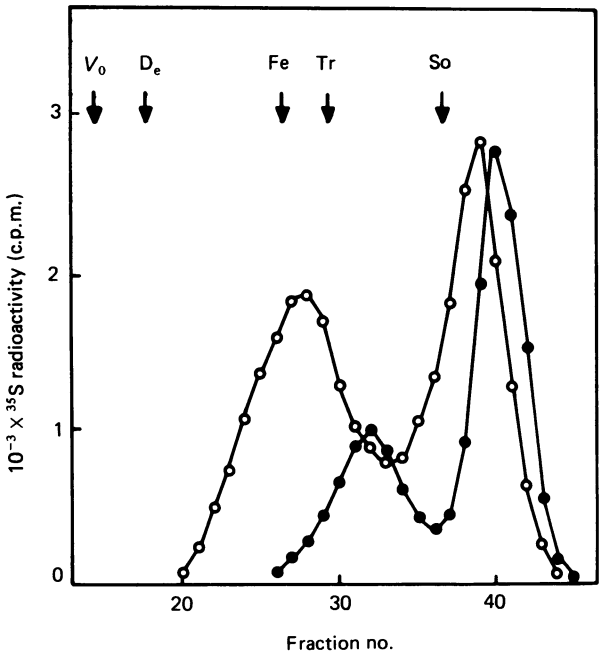

Fig. 3. Gel filtration of ^{35}S-labelled oligosaccharides as in Fig. 2

●, Fraction 5; ○, fraction 6.

RESULTS AND DISCUSSION

Carbohydrate composition and structural features of proteoglycan oligosaccharides

Glycopeptides prepared by Pronase digestion of [^{35}S]sulphate-labelled chondroitin sulphate proteoglycan were subjected to mild NaOH/$NaBH_4$ treatment, which converted approx. 50% of the glycopeptide mannose into mannitol (Krusius *et al.*, 1986). The ^{35}S-labelled oligosaccharides were fractionated by anion-exchange chromatography on DEAE-Sephadex with a linear gradient of pyridine/acetate buffer, pH 5.0 (Fig. 1). Eight well-separated fractions were obtained. Fractions 1, 2 and 4 contained no sulphate, and represented neutral, monosialyl and disialyl oligosaccharides respectively. Fractions 3, 5, 6, 7 and 8 contained [^{35}S]sulphate-labelled oligosaccharides, and fraction 9, which represented 50% of the [^{35}S]sulphate label, could be eluted only with 2 M-pyridine/acetate buffer. The isolated ^{35}S-labelled oligosaccharide fractions were analysed by gel filtration on Sephadex G-50 (Figs. 2 and 3). Fraction 9 oligosaccharides had the largest molecular size, and were eluted as a broad peak with a mean apparent M_r of 9500. The other oligosaccharide fractions were eluted in each case as two well-separated peaks. The apparent M_r values of both the large- and small-molecular-size fractions increased with increasing acidity (and [^{35}S]sulphate labelling) of the oligosaccharides. The carbohydrate composition of the fractions, and their M_r values obtained by gel filtration, are given in Table 1. Galactose and *N*-acetylglucosamine were the major sugars present, and carbazole-reactive material (listed as uronic acid in Table 1) was found only in the most acidic fraction. The relative amount of mannitol ranged from 0.4 to 0.8 mol/mol of 'oligosaccharide', and was not increased by repeating the $NaBH_4$ reduction. No other hexitols were present.

These data indicate that, in addition to oligosaccharides of M_r 800–1400 with a common GlcNAc(β1–3)-Manol core structure (eluted in Sephadex G-50 fraction II), the larger-molecular-size fractions (5-I to 8-I), having a mannose/mannitol molar ratio ranging from 2.7 to 4.8, probably also contain some asparagine-linked oligosaccharides. However, the considerably larger proportion of galactose and *N*-acetylglucosamine in these fractions and the approximately equal molar ratios of these two sugars suggest the presence of poly-*N*-acetyl-lactosaminyl disaccharide repeating units. These are evidently in the form of relatively short *O*-glycosidic mannose-linked keratan sulphate chains, since both [^{35}S]sulphate-labelled oligosaccharides in fractions 6-I to 8-I, and those labelled (predominantly as [^{3}H]mannitol) by alkaline-borotritide treatment of the proteoglycan glycopeptides (Krusius *et al.*, 1986), were depolymerized to the extent of 70–95% by treatment with endo-β-galactosidase. Methylation analysis demonstrated that galactose was mainly 3- and 6-*O*-substituted, and *N*-acetylglucosamine was predominantly 4,6-di-substituted (Table 2). These data support our conclusions that the larger oligosaccharides in DEAE-Sephadex

Table 1. Carbohydrate composition of proteoglycan-derived oligosaccharides isolated by anion-exchange chromatography (DEAE-Sephadex fractions 5–9) and gel filtration (Sephadex G-50 fractions I and II; see the legend to Fig. 2)

Fraction	Composition (mol/mol of oligosaccharide)								M_r	Total sugar (μmol/100 mg of protein)	^{35}S [c.p.m./nmol of (galactose +*N*-acetyl-glucosamine)]
	Mannitol	Mannose	Galactose	*N*-Acetyl-glucosamine	*N*-Acetyl-neuraminic acid	Fucose	Uronic acid	Total			
5-I	0.4	1.4	2.4	3.1	1.8	0.5	< 0.05	9.6	2100	4.88	800
5-II	0.8	0.2	0.9	1.2	0.8	0.1	< 0.05	4.0	800	10.04	1600
6-I	0.4	1.9	4.7	5.6	2.5	0.7	< 0.05	15.8	3100	5.23	1300
6-II	0.6	0.4	1.4	1.6	0.8	< 0.05	< 0.05	4.8	900	2.37	2400
7-I	0.6	1.6	5.3	6.1	2.9	0.7	< 0.05	17.2	3400	7.18	1200
7-II	0.8	0.4	1.7	2.3	1.0	< 0.05	< 0.05	6.2	1000	1.25	2300
8-I	0.7	2.4	7.5	9.4	2.6	0.9	< 0.05	23.5	4500	5.44	1500
8-II	0.7	0.4	1.8	1.8	1.8	0.3	< 0.05	6.8	1400	2.51	1400
9	0.7	2.2	17.0	16.2	3.8	2.3	2.8	45.0	9500	15.19	3300

fractions 6–8 contain a significant proportion of Gal(β1–4)GlcNAc-6-*O*-SO_4(β1–3) disaccharide repeating units in the form of short (M_r 3000–4500) keratan sulphate chains. Fraction 9 is almost entirely composed of longer keratan sulphate chains, of M_r 10000 (Krusius *et al.*, 1986).

F.a.b. m.s.

Studies of the smaller sulphated oligosaccharides by f.a.b. m.s. in the negative-ion mode demonstrated that the borodeuteride-reduced oligosaccharides of fraction 5-II yielded two major ions, at *m/z* 779 and 941. The ion at *m/z* 779 is compatible with an oligosaccharide containing a sialic acid, an *N*-acetylhexosamine, a hexitol, and $NaSO_3^-$ in place of one hydroxy-group proton, and the component at *m/z* 941, with a difference of 162 mass units, would contain an additional hexose residue. Negative-ion f.a.b. m.s. of borohydride-reduced oligosaccharides in fraction 6-II gave major ions at *m/z* 1333, 832 and 422, and somewhat less intense ions at *m/z* 1216 and 1166. The major ion at *m/z* 1333 would correspond to a disialylated disulphated pentasaccharide having the same sugar composition as the tetrasaccharide present in fraction 5-II (presumably mannitol, *N*-acetylglucosamine, galactose and sialic acid), whereas the ion at *m/z* 1166 is compatible with the monosulphated pentasaccharide having three less sodium atoms. Positive-ion f.a.b. m.s. of borohydride-reduced oligosaccharides in fraction 7-II showed several major ions at *m/z* 1021, 1043 and 1065, which are compatible with a structure containing a sialic acid, a hexose, an *N*-acetylhexosamine and a hexitol, together with two sulphate residues and one to three sodium atoms. Negative f.a.b. of the same sample showed a major ion at *m/z* 1041 (corresponding to the positive f.a.b. ion at *m/z* 1043), as well as less intense ions at *m/z* 1095 and 1009.

The M_r values and proposed sugar compositions of these oligosaccharides are in good agreement with the analytical data for the respective fractions presented in Table 1, and indicate that they contain sialylated and sulphated tri- and tetra-saccharides (fraction 5-II), disialylated, mono- and di-sulphated pentasaccharides (fraction 6-II), as well as a disulphated sialylated tetrasaccharide (fraction 7-II). It is likely that all of these oligosaccharides have a common GlcNAc(β1–3)Manol core structure. The considerably higher [^{35}S]sulphate specific radioactivity of fraction 6-II and 7-II oligosaccharides, compared with those in fraction 5-II (Table 1), is also consistent with the presence of an additional sulphate residue on many of the oligosaccharides in fractions 6-II and 7-II.

Molecular size of the proteoglycan

In our previous studies of the chondroitin sulphate proteoglycan, its molecular size could only be estimated from its gel-filtration behaviour in relation to a somewhat smaller heparan sulphate proteoglycan of M_r 220000 (Klinger *et al.*, 1985), for which calibration standards of appropriate size were available. However, Ohno *et al.* (1986) have since derived calibration relationships for chondroitin sulphate proteoglycan subunits that relate the hydrodynamic radius and the weight-average M_r (determined by light-scattering measurements) to the partition coefficient ($K_{av.}$) obtained by gel filtration on a column of Sepharose CL-2B. Although established on the basis of cartilage proteo-

Table 2. Relative amounts of differentially substituted galactose and *N*-acetylglucosamine residues in oligosaccharide fractions isolated by anion-exchange chromatography and gel filtration, after treatment of glycopeptides with $NaOH/NaBH_4$

The first number in the column headings refers to the respective fraction from the DEAE-Sephadex gradient, and I indicates the larger-molecular-size material (fraction I) present in the first of the two pools obtained from the subsequent Sephadex G-50 elution.

Component	Glycosidic linkage	Substituted residues isolated (mol/mol of oligosaccharide) Fraction 5-I	Fraction 7-I	Fraction 8-I
Galactose				
2,3,4,6-Tetra-*O*-Me	Terminal	0.1	0.1	0.2
2,4,6-Tri-*O*-Me	3	1.0	2.9	4.7
2,3,4-Tri-*O*-Me	6	1.0	1.8	2.0
2,4-Di-*O*-Me	3 and 6	0.3	0.5	0.6
N-Acetylglucosamine				
3,4,6-Tri-*O*-Me	Terminal	0.5	0.7	0.4
3,6-Di-*O*-Me	4	0.7	0.7	1.0
6-Mono-*O*-Me	3 and 4	< 0.1	< 0.1	< 0.2
3-Mono-*O*-Me	4 and 6	1.8	4.4	7.8

glycan subunits, the relationship of $K_{av.}$ to M_r was essentially linear down to the smallest (i.e. least glycosylated) species, which in this respect are very similar to the chondroitin sulphate proteoglycans of brain. Application of these calibration relationships to the gel-filtration data for several preparations of chondroitin sulphate proteoglycan from brain indicates an M_r of 260000, based on the elution peak for protein ($K_{av.} = 0.71$), and an M_r of 325000, based on the elution peak ($K_{av.} = 0.65$) for [^{3}H]glucosamine incorporated into hexosamine and sialic acid residues of the proteoglycan. The average of these values is very close to our earlier estimate of M_r 300000 used to calculate the relative proportions of the glycosaminoglycans and glycoprotein oligosaccharides (Krusius *et al.*, 1986).

We have previously shown that the larger molecules in the polydisperse population of brain chondroitin sulphate proteoglycans have almost twice as high a concentration of *N*-acetylglucosamine-containing glycoprotein oligosaccharides as those eluted in the subsequent fractions together with the protein peak (Kiang *et al.*, 1981). These data indicate that the larger chondroitin sulphate proteoglycan molecules, of M_r approx. 325000, are more highly glycosylated, owing to a greater concentration of mannose- and asparagine-linked oligosaccharides. The proteoglycan molecules eluted in the fractions containing the peak absorption at 280 nm, with an average M_r of 260000, contain twice the concentration of *N*-acetylgalactosamine-linked oligosaccharides and the same concentration of chondroitin sulphate as the larger molecules, whereas the proteoglycans of smallest size contain one-third the concentration of chondroitin sulphate and only 10–20% as many mannose- and asparagine-linked oligosaccharides as are present in the larger molecules (Kiang *et al.*, 1981).

Conclusions

It is known that proteoglycans from other sources contain both chondroitin sulphate and keratan sulphate polysaccharide chains, as well as *N*- and *O*-glycosidically linked glycoprotein oligosaccharides (De Luca *et al.*, 1980; Lohmander *et al.*, 1980; Nilsson *et al.*, 1982). However, the chondroitin sulphate proteoglycan of brain is distinguished by the presence of a large proportion of diverse, yet structurally related, mannose-linked *O*-glycosidic oligosaccharides, which have not yet been reported in other mammalian glycoproteins or proteoglycans. Preliminary studies indicate that, in the brain proteoglycan, these linkages are synthesized by a direct transfer of mannose from GDP-mannose, and do not involve the dolichol pathway (D. Shum, R. U. Margolis & R. K. Margolis, unpublished work).

Although mannosyl-*O*-serine/threonine linkages have not been described in other mammalian glycoconjugates, Spiro & Bhoyroo (1980) have reported the occurrence of glucuronosyl-(α1→6)-mannosyl-*O*-threonine linkages in cuticle collagen of the common clam worm (*Nereis virens*). Future studies of other tissues and species may reveal that oligosaccharides *O*-glycosidically linked via mannose residues are characteristic of specialized localizations or functions in a wider variety of complex carbohydrates.

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