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Use of large-scale hydrazinolysis in the preparation of *N*-linked oligosaccharide libraries: application to brain tissue

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In this report, we describe the preparation of a library of *N*-linked glycans from whole murine brain obtained by the large-scale hydrazinolysis of an acetone powder of the tissue followed by chromatographic procedures. 84% of the characterized oligosaccharides were found to be anionic, the remainder neutral. The anionic species were successively neutralized by neuraminidase (29%), aq. hydrofluoric acid (30%), and methanolysis (26%), indicating that approximately equal portions were sensitive to desialylation, dephosphorylation and desulfation, respectively. The presence of the sulfated fraction was confirmed by direct $^{35}SO_4$ metabolic labelling. A residual partially characterized fraction was found to be anionic through possession of carboxylic acid groups, unrelated to sialic acid. The purified oligosaccharides, in the absence of their original protein conjugates, were shown to retain those immunological characteristics essential for recognition by a specific monoclonal antibody, LS (412), that is known to recognize a carbohydrate epitope present on a number of neural adhesion molecules and functional in neural cell adhesion. These properties confirm the viability of scaling up the size of the hydrazinolysis procedure and adapting it to whole tissue for the production of glycan libraries and for the probing of structures of interest.

Keywords: *N*-linked, oligosaccharide library, L2/HNK-1 epitope, anionicity, murine brain, large scale hydrazinolysis

Abbreviations: ConA, concanavalin A; ELISA, enzyme-linked immunosorbent assay; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; g.u., glucose units; HRP, horseradish peroxidase; HVE, high voltage electrophoresis; Man, mannose; MS, mass spectrometry; N-CAM, neural cell adhesion molecule.

The accumulating evidence that oligosaccharides are directly involved in specific biological recognition processes [1–3] has led to increased interest in developments for their isolation and characterization, particularly for their provision to serve as tools or reagents in glycobiology.

In analytical studies, oligosaccharides have usually been isolated from purified glycoconjugates on a relatively small scale. *N*-linked glycans have been released from their protein conjugates by enzymatic methods (e.g., glycopeptidase F) or by the chemical method of anhydrous hydrazinolysis [4]. In this study, an alternative approach is described in which a larger scale 'library' of *N*-linked oligosaccharides is isolated from unpurified biological material, enabling subsequent probing and characterization to be made directly with the liberated glycans.

Brain tissue (murine) has been used since available evidence indicates that neural glycoproteins contain a wide variety of oligosaccharide classes [5, and references therein]. The method of hydrazinolysis was adapted to a larger scale for the release of *N*-linked glycans from the brain tissue preparation. It is anticipated that the nature and scale of production of oligosaccharide libraries in this way will assist studies of structural characterization in carbohydrate recognition. This may be illustrated by CNS glycoproteins, often available in purified form in relatively small quantities only, but in which the carbohydrate moieties are known to

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possess common epitopes functional in cell–cell and cell–matrix interactions, as detected through monoclonal antibody recognition [6–8].

Materials and methods

Extraction of brain tissue

Approximately 6 g lyophilized whole brains from adult mice of mixed ages, sexes and strains were homogenized in 150 ml cold acetone (−10 °C, AR grade) using an Ultra Turrax mechanical homogenizer (Janke & Kunkel, No. 6 setting, 3 min). The homogenate was filtered through Whatman No. 50 hardened paper and the residue was re-suspended in a further 150 ml cold acetone (−10 °C) and re-homogenized and filtered as before. The residue was dried under vacuum to remove residual acetone and then made into a slurry with 80 ml water and exhaustively dialysed against water at 4 °C. The sample was lyophilized and then cryogenically dried for a week over activated charcoal at −196 °C ($<10^{-6}$ bar).

Release and isolation of asparagine-linked oligosaccharides

Hydrazinolysis and re-N-acetylation. Asparagine-linked oligosaccharides were released by treatment with fresh, vacuum-distilled anhydrous hydrazine (40 ml, 25 °C), in a sealed glass vessel under argon. The reaction temperature was raised to 85 °C at 10 °C h^{-1} and then maintained at 85 °C for a further 12 h. Hydrazine was removed by evaporation under reduced pressure followed by repeated co-evaporation with anhydrous toluene. Re-*N*-acetylation of the oligosaccharides was carried out with a fivefold molar excess of acetic anhydride in saturated $NaHCO_3$ for 10 min at 0 °C and then 50 min at room temperature. The total molarity of amino groups in the hydrazinolysate was included in the estimation, assuming brain dry weight to be 50% protein [9]. Sodium ions were removed by passage through Dowex AG-50 X12 (H^+ form) and the water eluate was reduced in volume by rotary evaporation at 27 °C and then lyophilized.

Purification of oligosaccharides. The preparation was applied to a column of microcrystalline cellulose (E. Merck, Darmstadt, Germany) 1.5 cm × 10 cm equilibrated in 1-butanol–ethanol–water, 4:1:1 by vol. During application the column flow was stopped and the sample was applied, dissolved in water (1 vol) and mixed with ethanol (1 vol) on the surface of the cellulose matrix. 1-Butanol (4 vol) was immediately added and also mixed with the sample components. Peptides were eluted away by 1-butanol–ethanol–water, 4:1:1 by vol and the oligosaccharides were recovered by elution with water after using methanol as transition solvent. The sample was then reduced in volume by evaporation at 27 °C, lyophilized and re-dissolved in a fivefold molar excess of 10 mM cupric acetate at 25 °C for 45 min to cleave any residual oligosaccharide hydrazides. After passage through a tandem column of Chelex 100 (Na^+) and Dowex AG50W-X12 (H^+) the oligosaccharide eluant was lyophilized and then re-chromatographed on microcrystalline cellulose, as above. The oligosaccharide-containing fraction was then filtered through a 0.45 μm Teflon filter (Millex SR) and re-lyophilized.

Bio-Gel chromatography. For preparative work, the majority of the preparation was subjected to Bio-Gel P-4 gel permeation chromatography as described by Ashford *et al.* [10]. The voided material (Vo) of the Bio-Gel P-4 chromatography water eluate contained all acidic oligosaccharides, separated from neutral oligosaccharides that were retarded by the column according to size. The numerical superscripts on the P-4 chromatograms refer to the elution position of glucose oligomers in glucose units (g.u.) as detected by a refractive index monitor.

The initial voided acidic material was dried and re-suspended in 50 mM sodium acetate (pH 6.0, BDH, Aristar) and applied to a Bio-Gel P-2 (200–400 mesh) gel permeation chromatography column (1.5 cm × 100 cm) using 50 mM sodium acetate (pH 6.0, containing chlorbutol, 1 mg ml^{-1}). The eluant was monitored for refractive index as above. Fractions eluting at the column void (and unresolved from it) were separated from smaller anionic species subsequently retarded by the column. The preparation was desalted on Dowex AG50W-X12 (H^+) and lyophilized.

Neutralization of oligosaccharides

Sequential neutralization procedures were used. In the first instance, *Arthrobacter ureafaciens* neuraminidase, (Calbiochem) was used at 10 U ml^{-1} in 0.1 M sodium acetate pH 5.0 (37 °C, 18 h) to remove α2-3, α2-6 and α2-8 linked sialic acids.

Removal of mono- or diphosphate esters was then achieved by incubating the oligosaccharides with 50% aqueous HF (Pierce) on a water–ice slurry at 0 °C for 48 h [11]. The pH of the ice-cold sample was adjusted to 6.0 by addition to a titrated volume of frozen LiOH (Sigma) solution. After thawing and centrifugation, the supernatant was removed and the LiF pellet was washed. The sample was dried and then re-*N*-acetylated as above.

Finally, removal of sulfate esters was achieved by lyophilization of the oligosaccharides, suspension in 50 mM HCl in anhydrous methanol and incubating in a sealed tube at room temperature for 15 h. The samples were dried under vacuum and re-*N*-acetylated as above. Following re-*N*-acetylation, aliquots were treated with 50 mM NaOH at 50 °C for 2 h to remove any methyl esters of carboxylic acid groups.

These procedures were used in both preparative and analytical studies. Preparative separation of newly neutralized oligosaccharides from resistant anionic species was by Bio-Gel P-4 gel permeation chromatography as above.

Analytical studies

For analytical work, aliquots of the total oligosaccharide preparation were radiolabelled by reduction with NaB^3H_4 at pH 11.0 [10]. Reduced acidic oligosaccharides were separated from neutral ones by high-voltage electrophoresis at pH 5.4 in pyridine–acetic acid–water, 3:1:387 by vol [10]. Detected fractions were eluted with water, and neutral oligosaccharides, which had remained at the origin, were subjected to Bio-Gel P-4 chromatography (as above), the effluent being monitored by a Berthold HPLC radioactivity flow monitor (model LB503, Lab-Impex). Reduced terminal monosaccharides (^3H labelled), released by acid hydrolysis (see legend to Fig. 2), were separated by high voltage paper electrophoresis in 60 mM sodium tetraborate buffer, pH 9.5 (Whatman No. 1 paper, 8.5 kV m^{-1}) in a Locarte electrophoresis unit [10]. [1-^{3}H]-2-deoxyribitol was used as an internal calibration standard.

Oligosaccharide quantitation

For quantitative analysis of oligosaccharide fractions used in the competitive ELISA study, 2–10 nmol (with 1 nmol scylloinositol as internal standard) were reacted with anhydrous methanolic HCl (0.5 M) at 80 °C (18 h). After re-*N*-acetylation (with 50 µl pyridine and 10 µl acetic anhydride) and drying, TMS (trimethylsilyl) derivatives were prepared by the addition of 25 µl Sigma-Sil A, and aliquots were injected onto a CP-SIL 8 capillary column (0.25 mm × 30 m, Supelco), with helium as carrier gas, for GC/MS analysis (Hewlett-Packard 5996C instrument) with flame ionization detection and a Hewlett-Packard integrator (model 3393A) to record GC data. The GC oven temperature programme was held at 90 °C for 1 min, then 90 °C to 140 °C at 30 °C min^{-1}, 140 °C to 180 °C at 4 °C min^{-1}, 180 °C to 240 °C at 10 °C min^{-1}.

The mannose peak areas obtained above (relative to the internal standard) were used to estimate the number of moles of oligosaccharide by reference to the mannose peak areas derived from known quantities of the external standard: Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)-Manβ4GlcNAcβ4GlcNAc. The estimation is approximate since it is based on the assumption that each mole of oligosaccharide in a fraction will contain, on average, three mannose residues.

Competitive ELISA

Competitive ELISA assays were performed by pre-incubating the first antibody, the rat monoclonal L2 antibody 336, with the competitor, the oligosaccharide preparation under test, for 30 min at 37 °C. The antibody was used at a concentration that corresponded to 50% of its maximum value as determined by antibody dilution measurements. The pre-incubated L2 antibody [12] and competitor oligosaccharide were then added as a mixture to PVP microtitre plates that had been coated with total L2 glycoproteins (0.1 µg ml^{-1} in 0.1 M $NaHCO_3$) from adult mouse brain [12] for 3 h at 20 °C. Plates were washed and developed using an anti-rat IgG antibody conjugated with HRP as the second antibody and ABTS reagent (acetate buffer containing 2,2′-azino-di-3-ethylbenzthiazolin sulphonate). The absorbance was read at 405 nm. L2 total glycoproteins were isolated from detergent extracts of adult mouse brains (from mixed ages, sexes and strains) over affinity columns containing monoclonal antibodies 336 or 412 [12, 13]. L1 and N-CAM from adult mouse brain [14] were also used as antigens to coat the plastic wells.

Preparation of ^{35}S-labelled murine brain oligosaccharides

^{35}S-metabolically labelled oligosaccharides were obtained by intracerebral ventricular injection of 25 µCi sodium [^{35}S]sulphate (Amersham International, UK) in 1 µl 0.85% (w/v) NaCl into the 3rd ventricle of each of 40 mice (BALB/c strain, 20 male, 20 female). The animals were left for 18 h before killing and removal of the whole brain. The brains were prepared and the oligosaccharides isolated as described above.

Results

Oligosaccharide reducing terminal analysis

Analysis of the monosaccharides at the reducing terminal of the oligosaccharides revealed that this position was essentially occupied by *N*-acetylglucosamine (Fig. 1) typical for *N*-linked oligosaccharides. *N*-Acetylgalactosamine was detectable in trace amounts. No other monosaccharides were positively identified.

Oligosaccharide fractionation

When the total oligosaccharide preparation that had been radiolabelled by reduction with NaB^3H_4 was subjected to HVE (in pyridine–acetate buffer, pH 5.4) a complex profile was obtained (Fig. 2, upper panel). Elution and counting of the radioactive oligosaccharides showed that almost 20% were neutral and remained at the origin, while more than 80% were acidic and migrated, being negatively charged. When the same preparation of total tissue oligosaccharides, in its nonreduced state, was subjected to Bio-Gel P-4 permeation chromatography in water, the majority of glycans eluted with the column void (Vo) (Fig. 2, lower panel). In the absence of buffers, anionic species are repelled by the column and elute in this fraction. A small proportion was retarded by the column, and its profile is enlarged in Fig. 2, lower panel, inset, representing naturally occurring neutral oligosaccharides with various hydrodynamic volumes of 8–20 g.u.

The naturally occurring acidic oligosaccharides, after radiolabelling by reduction to increase sensitivity of detection, were digested with neuraminidase and subsequently re-fractionated by Bio-Gel P-4 permeation chromatography. This yielded both an acidic fraction that was

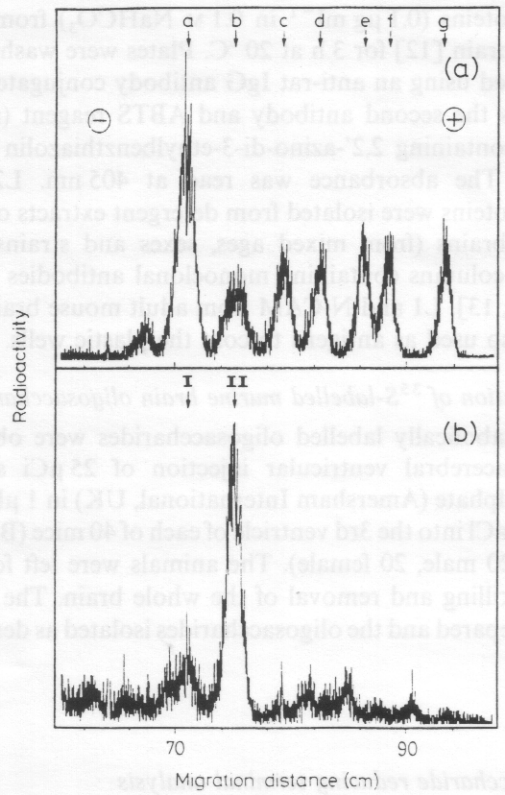

Figure 1. High voltage radioelectrophoretogram of reducing terminal monosaccharides from murine brain total oligosaccharide preparation: (a) standards, and (b) murine brain. Tritium-reduced oligosaccharides from the total preparation were incubated in 1 M HCl (Pierce, Sequanal Grade) at 100 °C for 2 h. After removal of the acid, re-*N*-acetylation and desalting, the residue was subjected to a second round of hydrolysis and re-*N*-acetylation and mixed with tritium-reduced 2-deoxyribose as internal standard. Electrophoresis was in 60 mM sodium tetraborate buffer, pH 9.5 (see the Materials and methods section). Labelled peaks I and II were identified as *N*-acetylgalactosaminitol and *N*-acetylglucosaminitol, respectively, according to a parallel track containing the following tritium-labelled standards: (a) *N*-acetylgalactosaminitol, (b) *N*-acetylglucosaminitol, (c) xylitol, (d) glucitol, (e) mannitol, (f) fucitol and (g) galactitol.

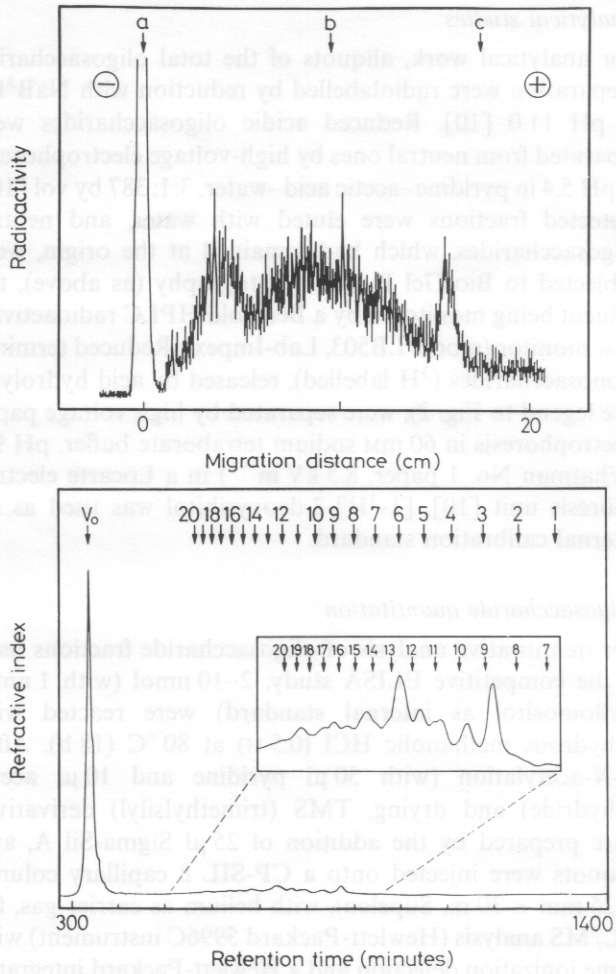

Figure 2. Analysis of total hydrazine-released oligosaccharides from murine brain. Upper panel: high voltage radioelectrophoretogram in pyridine–acetate buffer, pH 5.4 (see the Materials and methods section). The migration positions are indicated for the standard saccharides: (a) lactitol and (b) sialyllactitol, and (c) for the dye Bromophenol Blue. Lower panel: Bio-Gel P-4 (~400 mesh) gel permeation chromatogram (see the Materials and methods section). Detection was by change of refractive index (RI); oligosaccharides were not reduced. Inset: expanded scale for RI of included oligosaccharides. The numerical superscripts refer to glucose units (see the Materials and methods section).

resistant to neuraminidase and a neutral fraction. The latter is shown in Fig. 3(a), generated from those oligosaccharides that were originally sialylated and not possessing any other anionic species. It can be seen that these desialylated neutral glycans have proportionately larger hydrodynamic volumes than the oligosaccharides that were naturally neutral (Fig. 2, lower panel, inset).

Further neutralization of the neuraminidase-resistant fraction was made by digestion with aqueous HF (50%) and then re-fractionating by Bio-Gel P-4 chromatography as before (vide supra). The HF-resistant acidic fraction eluted in the void volume and the neutral fraction generated by the dephosphorylation action of aqueous HF are shown as a separate chromatographic run in Fig. 3(b). Again, the large envelope of peaks between 7 and 17 glucose units (bar Fig. 3(b)) is typical of *N*-linked oligosaccharides. When this envelope is digested with endoglycosidase H, extensive cleavage occurs within the *N*,*N*′-diacetylchitobiotol core releasing the terminal *N*-acetylglucosaminitol (2.5 glucose units; data not shown), indicating that the majority of these phosphorylated structures were of oligomannose or hybrid class.

The HF-resistant acidic fraction was digested with methanolic HCl for desulfation (and base-treated, see the Materials and methods section, to remove methyl esters of any residual carboxylic acid groups) and the desalted sample was subjected to HVE at pH 5.4 (see the Materials and methods section). The profile obtained is shown in Fig. 4(a),

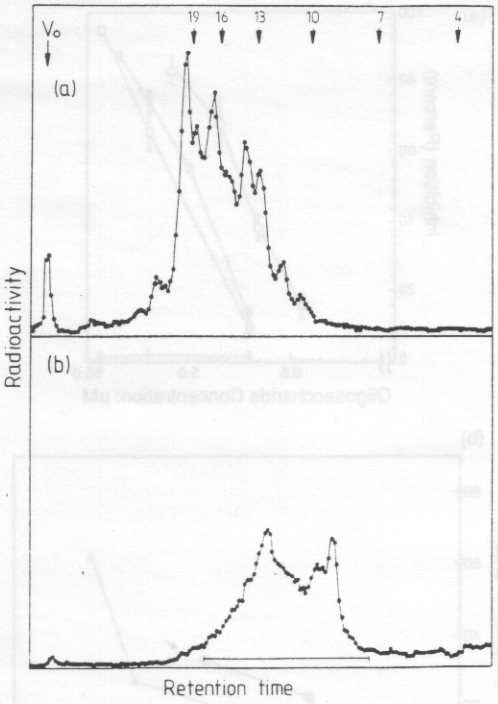

Figure 3. Bio-Gel P-4 (~ 400 mesh) gel permeation chromatograms of murine brain oligosaccharides neutralized by sequential deacidification procedures. Detection was by radiolabelling of the reduced oligosaccharides. The numerical superscripts refer to glucose units (see the Materials and methods section) Vo = void. (a) Neutrals, 12–20 g.u. generated by neuraminidase (*Arthrobacter ureafaciens*). (b) Neutrals 7–17 g.u. (indicated by bar) generated by aqueous HF (after neuraminidase treatment). Resistant acidic oligosaccharides were not chromatographed in either (a) or (b), (the small quantity of label eluting near the void volume could represent large glycans or residual 'blank' material).

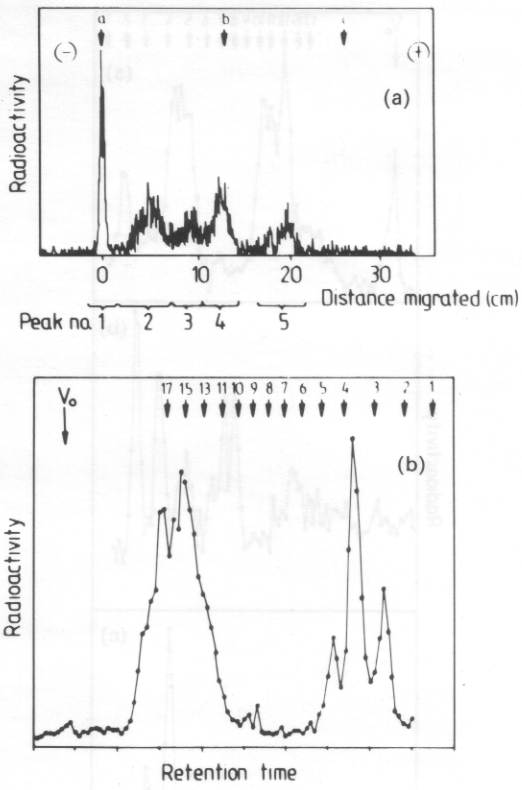

Figure 4. Digestion of neuraminidase and aqueous HF-resistant acidic glycans by methanolysis (and base treatment). (a) High voltage electrophoresis at pH 5.4, as described in the Materials and methods section. Relative % distribution of radioactivity: peak 1, 30.4%; peak 2, 18.2%; peak 3; 12.9%; peak 4, 28.1%; peak 5, 10.4%. Standard markers a, b and c are as for Fig. 2. (b) Bio-Gel P-4 gel permeation chromatogram of peak 1 from HVE of (a). Notations as for Fig. 3..

with five major peaks visible. It is seen that approximately one third of the radiolabel at this stage behaves as neutral oligosaccharitols (peak 1) by remaining at the origin on the HVE paper. This is confirmed by the fact that when the radiolabel of peak 1 is eluted and subjected to Bio-Gel P-4 chromatography, the material is retarded on the column, giving the profile shown in Fig. 4(b).

The methanolysis procedure did not cause degradation of standard neutral biantennary oligosaccharides, but did not give complete desulfation of standard sulfated sugars (unpublished observation, and see [15]). A second methanolysis, followed by the base treatment, was therefore carried out on each of the four peaks (Nos 2–5 inclusive) after elution from the HVE paper. With the exception of the most mobile peak (No. 5), which remained resistant to further methanolysis, another quarter of the remaining radiolabel then behaved as neutral oligosaccharitols, on further high voltage electrophoresis. The profiles of these three neutral fractions on Bio-Gel P-4 gel permeation chromatography are shown in Fig. 5.

The two sequential methanolyses effectively neutralized half of the acidic material that had resisted the neuraminidase and aq. HF treatments. The fractions of the radiolabelled peaks (Nos 2–5 inclusive) that were not neutralized by these procedures were subjected to a third methanolysis followed by high voltage electrophoresis without prior base treatment. Between 40% and 50% of the remainder of each fraction then remained at the origin as if neutralized. Elution of these 'neutral' fractions, followed by base treatment and Bio-Gel P-4 gel permeation chromatography revealed profiles dominated by peaks at the void of the column (data not shown). The previous methanolysis and base treatment had indicated that there was very little neutral material large enough to be voided on Bio-Gel P-4, and so the peaks are consistent with the presence of acidic oligosaccharides containing carboxylic acid groups that had earlier been esterified. Small quantities of neutral oligosaccharides (i.e., retarded on the Bio-Gel P-4 column) were observed from peaks 2 and 4, consistent with further slight residual desulfation of some oligosaccharides.

The relative percentage composition of murine brain *N*-linked oligosaccharide classes, is indicated by the procedures described above. 84% of the characterized

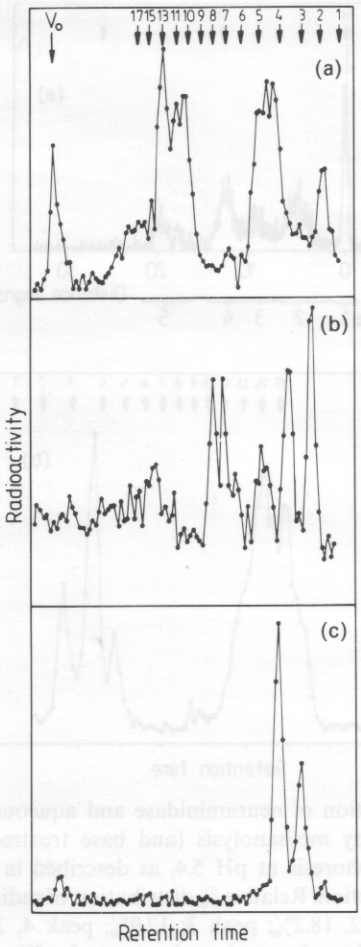

Figure 5. Repeat methanolysis and base treatment of resistant acidic glycans. Bio-Gel P-4 gel permeation chromatograms of neutral fractions; separated by HVE at pH 5.4, generated by second methanolysis (and base treatment) of peaks 2–5 from HVE of Fig. 4(a). The profile for peak 2 is shown in (a), for peak 3 in (b) and for peak 4 in (c). Note that there was no neutral fraction generated for chromatography from peak 5.

oligosaccharides were anionic, the remainder neutral. The anionic species were successively neutralized by neuraminidase (29%), aqueous HF (30%) and methanolysis (26%), indicating that approximately equal proportions were sensitive to desialylation, dephosphorylation, and desulfation, respectively. Approximately half the residual oligosaccharides were assigned carboxylic acid groups as their anionic moiety, distinct from the sialylated glycans.

Determination of the L2/HNK-1 containing carbohydrate fraction

Oligosaccharide fractions were probed for the presence of carbohydrate epitopes recognized by the L2 monoclonal antibody. The results of the competitive ELISA study are shown in Fig. 6(a,b). Extracts of a crude fraction of adult mouse brain *N*-linked oligosaccharides showed inhibition of antibody binding to the total L2 glycoproteins. On

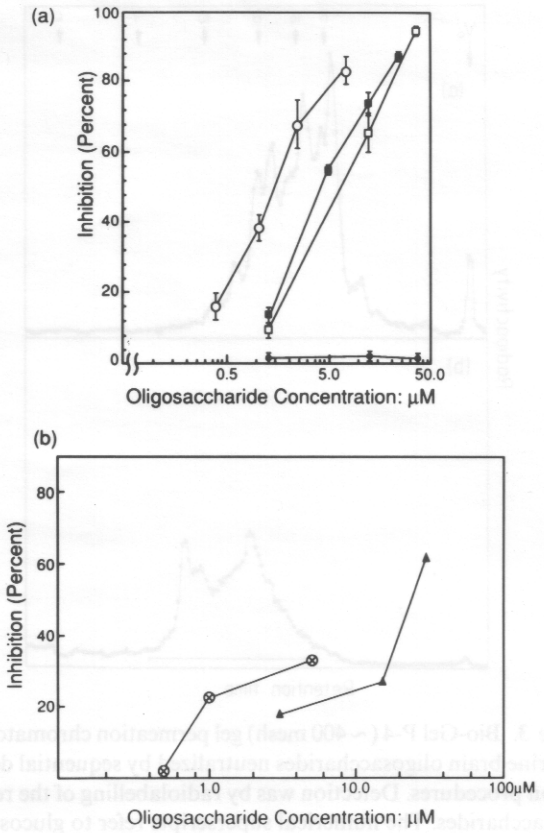

Figure 6. Competitive ELISA showing percentage inhibition of monoclonal L2 antibody 336 binding to L2-positive glycoproteins by oligosaccharide fractions from adult murine brain. The L2 (336)-reactive glycoproteins were obtained from detergent-extracts of murine brain membranes. (a) Oligosaccharide fractions were □, total; ■, natural acidic; ●, natural neutral; and ○, neuraminidase-resistant acidic. See the Materials and methods section for details, including quantification of oligosaccharides. Potency ratio for oligosaccharide concentration giving 50% inhibition: neuraminidase-resistant acidic/natural acidic = 0.36. (b) In a separate experiment, acidic oligosaccharides resistant to both neuraminidase and aqueous HF, ⊗, were compared with the natural acidic fraction, ▲. Estimated potency ratio for oligosaccharide concentration giving 50% inhibition: neuraminidase and aqueous HF-resistant acidic/natural acidic = 0.28.

separation into its constituent acidic and neutral fractions, all of the inhibitory effects remained with the acidic glycans, no inhibition being observed with the neutrals over a wide range of oligosaccharide concentrations. As a result, the specific inhibitory capacity on a molar basis was slightly greater for the acidic than for the total unfractionated preparation.

The neuraminidase-resistant acidic fraction showed a greater potency for inhibiting the antibody–antigen recognition, the required concentration at 50% inhibition being approximately 2.5 μM. The neutral glycans generated by neuraminidase showed no inhibitory effects (data not shown) similar to the naturally occurring neutrals.

In further experiments, the inhibitory effects were seen to

Table 1. Competitive ELISA showing percentage inhibition of binding of various monoclonal antibodies to corresponding antigens by the murine brain acidic oligosaccharide fraction resistant to neuraminidase[a].

Monoclonal antibody	*Antigen*			
	L1	*N-CAM*	*L2*[b] from *336 column*	*L2*[b] *from 412 column*
L2 (336)	Not done	Not done	73%*	70%
L2 (412)	64%*	38%	69%	74%
L1 (559)	1%**	Not done	−3%	4%
N-CAM (H28)	Not done	−2%**	0%	8%

[a] Concentration of oligosaccharide: *, 2.5 μM; or **, 7.5 μM, where indicated.
[b] See the Materials and methods section.

persist in the fraction remaining anionic after both neuraminidase and aqueous HF treatments (Fig. 6(b)) and with increased specificity according to the normalized concentration potency. These results are consistent with the epitope being present in sulfated oligosaccharides.

To evaluate the specificity of the competitive inhibition of the binding of the L2 antibody to its antigenic glycoproteins, other monoclonal antibodies and antigens were tested in the same system using the neuraminidase-resistant acidic fraction of glycans. It can be seen (Table 1) that greatest specificity existed between either of the L2 monoclonal antibodies and their respective antigens. Significant inhibition was also seen for the L2 (412) antibody binding to L1 and N-CAM since these glycoproteins have sub-populations carrying the L2 carbohydrate epitope.

The oligosaccharide preparation gave little or no inhibition to the binding of N-CAM or L1 559 monoclonal antibodies, which is to be expected since they are known to react with the protein moiety of their respective antigens.

$^{35}SO_4$ *labelling of brain oligosaccharides in vivo*

A large percentage (26%) of brain oligosaccharides were neutralized by methanolysis (after resisting neutralization by neuraminidase and aqueous HF, see above) and were therefore consistent with being sulfated (see the Discussion section). This fraction also possessed the greatest specificity for inhibition of L2 monoclonal antibody binding by competitive ELISA. It was therefore of importance to establish directly that sulfated oligosaccharides were present in the library. For this purpose, metabolic labelling of brain oligosaccharides with $^{35}SO_4$ was achieved *in vivo* through intra-cerebral ventricular injection (see the Materials and methods section).

A Folch extract of an aliquot of the isolated brain oligosaccharides resulted in partitioning of all radioactivity into the aqueous phase. Furthermore, there was no migration of radioactivity when the sample was subjected to

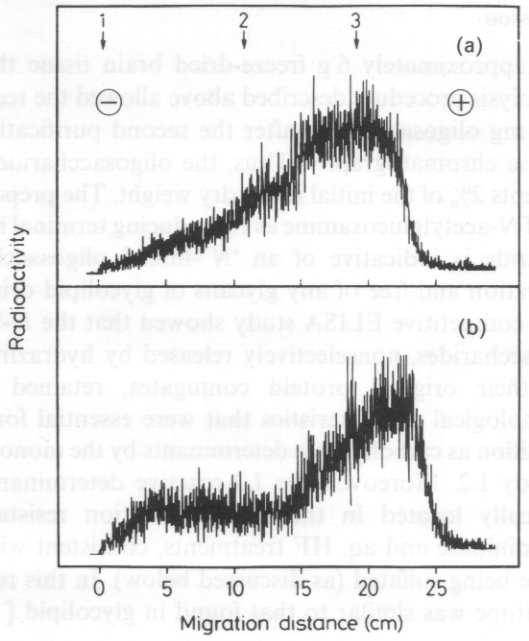

Figure 7. High voltage radioelectrophoretogram of *in vivo* ^{35}S-labelled murine brain oligosaccharides. (a) Naturally occurring. Electrophoresis was in pyridine–acetate buffer, pH 5.4 (see the Materials and methods section). The migration positions are indicated for lactitol (1), sialyllactitol (2) and Bromophenol Blue (3). (b) As part (a), but with the oligosaccharides pre-treated with neuraminidase (*Arthrobacter ureafaciens*). Enzyme concentration, 10 u ml^{-1} in 0.1 M sodium acetate buffer at pH 5.0, 18 h, 37 °C.

descending paper chromatography in 1-butanol–ethanol–water, 4:1:1 by vol, for 24 h, indicating that the ^{35}S label was associated with carbohydrate larger than a trisaccharide. In addition, analysis of the reducing terminal monosaccharide (after NaB^3H_4 reduction, acid hydrolysis and borate HVE, pH 9.5) showed that only GlcNAc-ol was found (similar to Fig. 1), confirming that this preparation was one of *N*-linked oligosaccharides.

High voltage paper electrophoresis of the total ^{35}S-labelled population showed a highly heterogeneous and mobile species (Fig. 7(a)). This profile may be contrasted with that of the ^3H-reduced oligosaccharides (Fig. 2) representing all glycans present in molar proportions. It is clear from the ^{35}S profile that sulfated species were present that carry multiple anionic groups, since the position of the Bromophenol Blue marker is typical for that of a disialyl-biantennary complex type of oligosaccharide. Treatment of the ^{35}S preparation with neuraminidase or aqueous HF resulted in some loss of mobility of the oligosaccharide, but much remained very mobile and heterogeneous (see Fig. 7(b), for example, for [^{35}S]oligosaccharides after neuraminidase treatment). It is therefore concluded that sulfated oligosaccharides were a significant class of glycans in this murine brain library and that a few species were also sialylated or phosphorylated.

Discussion

From approximately 6 g freeze-dried brain tissue the hydrazinolysis procedure described above allowed the recovery of 120 mg oligosaccharide after the second purification by cellulose chromatography. Thus, the oligosaccharide yield represents 2% of the initial tissue dry weight. The preponderance of *N*-acetylglucosamine as the reducing terminal monosaccharide is indicative of an '*N*'-linked oligosaccharide preparation and free of any glycans of glycolipid origin.

The competitive ELISA study showed that the *N*-linked oligosaccharides, nonselectively released by hydrazinolysis from their original protein conjugates, retained those immunological characteristics that were essential for their recognition as carbohydrate determinants by the monoclonal antibody L2. Moreover, the L2-reactive determinant was specifically located in the anionic fraction resistant to neuraminidase and aq. HF treatments, consistent with the epitope being sulfated (as discussed below). In this respect, the epitope was similar to that found in glycolipid [16].

Oligosaccharide classes

The 'library' of *N*-linked oligosaccharides from murine brain was dominated by anionic classes that were heterogeneous in character. The main fractionation procedures depended analytically on the sequential neutralization of these anionic species and their separation by HVE. This resulted in several groups of glycans being defined: (i) naturally neutral, (ii) those with neuraminidase-sensitive sialic acid as the only anionic moiety (sialylated), (iii) oligosaccharides neutralized by HF treatment (phosphorylated), following desialylation, (iv) those neutralized by methanolysis and base treatment (sulfated), after neuraminidase and HF treatment, and (v) those neutralized by methanolysis but anionic again after base treatment (containing carboxylic acid groups not related to sialic acid).

(i) *Naturally neutral.* The Bio-Gel P-4 chromatography profile (Fig. 2 inset) was very similar to that reported for the Thy-1 glycoprotein from rat brain [17] which contained oligomannose, hybrid and complex structures.

(ii) *Neuraminidase-sensitive sialic acid as the only anionic moiety.* This group of oligosaccharides, neutralized by neuraminidase, may have contained α2-3, α2-6 and α2-8 linked sialic acids because of the specificity of the *Arthrobacter ureafaciens* enzyme. The overall size of these desialylated oligosaccharides was typical of complex-type glycans.

(iii) *Neutralized by HF treatment (after neuraminidase).* Although acid-resistant phosphodiesters have been reported as substituents of oligosaccharides (e.g. [18]) 50% aqueous hydrogen fluoride was used in this study as previously it had removed successfully phosphodiesters from oligosaccharides [11]. This acid was found not to degrade a standard biantennary complex oligosaccharide, as tested by Bio-Gel P-4 chromatography, and not to remove sulfate esters (analysis of glucose 6-sulfate and *N*-acetylglucosamine 3-sulfate by GC/MS, unpublished observation). The oligosaccharides neutralized in this group therefore contained phosphoesters either alone or in combination with sialic acid residues. Additional tests using mild hydrolysis with acetic acid [19] to remove any neuraminidase-resistant sialic acids and alkaline phosphatase to remove phosphomonoesters allowed further subfractionation [20]. The large percentage of phosphorylated oligosaccharides (30%) recovered was unexpected but may have been derived in part from intracellular glycoproteins. The phosphorylated oligosaccharides appeared as smaller oligosaccharides (compared with the sialylated or sulfated oligosaccharides) consistent with oligomannose or hybrid type structures. This was supported by extensive cleavage with endoglycosidase H.

(iv) *Neutralized by methanolysis and base treatment (after neuraminidase and HF treatment).* A variety of methods were studied for chemical desulfation and methanolysis proved to be the most efficient with complex saccharides (unpublished observation). Methanolysis did not degrade a standard complex biantennary oligosaccharide and less than 10% of core fucose [Fuc(α1-6)GlcNAc-ol] was hydrolysed, and only 2.5% of sialic acid from 3′(6)′-sialyllactitol. It was found that sugars possessing carboxylic groups were rendered neutral by methanolysis, indicating the formation of methyl esters: these were hydrolysed by base treatment. Oligosaccharides in the fourth group were therefore sulfated, with or without the presence of the other anionic species. A large majority was taken to have possessed only the sulfated anionic group since methanolysis (which does not effectively de-sialylate) of the naturally occurring total anionic fraction generated 85% of those neutrals that were generated by the methanolysis treatment when carried out after the neuraminidase and aqueous HF stages [20]. This is supported by the relatively small change seen in the HVE profile after the ^{35}S-labelled oligosaccharide preparation was treated with neuraminidase (Fig. 7(a,b)).

The high incidence of sulfated oligosaccharides, neutralized by methanolysis (26%), is interesting in the light of such glycans being participants in cell adhesion (e.g., N-CAM carried sulfated N-glycans, [8, 12, 21]). Both *N*- and *O*-sulfate esters have been reported on *N*-linked glycans [22]. Interestingly, although the majority of oligosaccharides that were readily desulfated were 11–19 glucose units in size (Fig. 4(b)), smaller saccharides were also found. Similar patterns were observed for the sulfated oligosaccharides present on the cell surface of *Dictyostelium discoideum* [23], and these were shown to be developmentally regulated.

(v) *Neutralized by methanolysis, but anionic again after base treatment.* The properties of this oligosaccharide fraction indicate the presence of carboxylic acid groups, distinct from sialic acid, as the anionic moiety. Such groups, in uronic acids such as glucuronic, would have resisted earlier

neutralization procedures and possibly be contained in any glycosamino-glycan-like or related structures [22, 24, 25]. The complexity of this class is illustrated by the resolution of four major fractions by HVE alone.

In conclusion, the large-scale hydrazinolysis procedure applied to tissue preparations is a convenient strategy for the provision of oligosaccharides as representative tissue glycan libraries. The partial characterization of the oligosaccharides, as illustrated with neural tissue for a wide variety of glycan classes, supports the viability of the preparation and its potential value as a library from which carbohydrate epitopes of interest may be probed and analysed.

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