Journal of Neurochemistry
Raven Press, Ltd., New York

Detection of Multisulphated N-Linked Glycans in the L2/HNK-1 Carbohydrate Epitope Expressing Neural Adhesion Molecule P_0

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Abstract: P_0, the most abundant glycoprotein of PNS myelin, is a homophilic and heterophilic adhesion molecule. P_0 is known to contain a glycoform population that expresses the L2/HNK-1 carbohydrate epitope found on other neural adhesion molecules, and to be functionally implicated centrally in neural cell adhesion and neurite outgrowth. This carbohydrate epitope has been characterized previously from glycolipid structures and contains a sulphated glucuronic acid residue. However, the L2/HNK-1 carbohydrate epitope has not been characterized in glycoproteins. Because P_0 possesses only one glycosylation sequon, the number of P_0 glycoforms is equal to the heterogeneity of the glycan species. Here we report that the carbohydrate analysis of L2/HNK-1-reactive P_0 showed the presence of anionic structures containing sialic acid and sulphate in various combinations. At least one sulphate residue was present in 80% of the monosaccharide sequences, and 20% contained three sulphates. High-resolution P4 gel chromatography of the desialylated and desulphated oligosaccharides showed substantial heterogeneity of monosaccharide sequences. Sequential exoglycosidase digestions indicated that the majority of the structures were of the hybrid class, although the sulphated structures were found to be endoglycosidase H-resistant. **Key Words:** Glycoprotein—P_0—Carbohydrate epitope—Sulphated glycans—Neural adhesion molecule. **Field M. C. et al.** Detection of multisulphated N-linked glycans in the L2/HNK-1 carbohydrate epitope expressing neural adhesion molecule P_0. *J. Neurochem.* **58,** 993–1000 (1992).

The PNS-specific glycoprotein P_0 accounts for most of the glycoprotein present in purified peripheral myelin (Greenfield et al., 1973). P_0 has an apparent molecular mass of 28–30 kDa and is one of the smallest members of the immunoglobulin superfamily (Lemke and Axel, 1985; Sakamoto et al., 1987). P_0 has recently been shown to act as both a homophilic (Filbin et al., 1990; Schneider-Schaulies et al., 1990) and a heterophilic adhesion molecule (Schneider-Schaulies et al., 1990).

P_0 undergoes a number of posttranslational modifications, including glycosylation (Asn^{93}), acylation (site unknown), and in situ phosphorylation, within the myelin sheath (see Poduslo, 1989). Sulphation of the oligosaccharide chain of P_0 occurs in myelin assembly during development, and ceases in the adult (Poduslo, 1989). Sulphation of P_0 also occurs after nerve crush injury, but not after permanent transection of adult sciatic nerve in the rat (Poduslo, 1989). P_0 metabolically labelled with sulphate incorporates radioactivity that can be released from the protein using the enzyme *N*-Glycanase (Poduslo, 1989). Sulphation of P_0 can be inhibited by incubating cells with the glycosidase-processing inhibitors deoxymannojirimycin and, to a lesser extent, swainsonine (Poduslo, 1989).

P_0 isolated from the PNS expresses various carbohydrate structures, among them the L2/HNK-1 and L3 carbohydrate epitopes (Bollensen and Schachner,

Received February 19, 1991; revised manuscript received July 4, 1991; accepted August 6, 1991.

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Abbreviations used: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; HVE, high-voltage paper electrophoresis; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; g.u., glucose units.

1987; Martini et al., 1988). The L2/HNK-1 carbohydrate is expressed by several neural recognition molecules and has been shown to be involved in cell interactions (Kunemund et al., 1988), and its structure has been determined on cross-reacting glycolipids where sulphated glucuronic acid is thought to be an important determinant (Chou et al., 1985, 1986, 1987; Noronha et al., 1986; Ariga et al., 1987; Chou and Jungalwala, 1988). The L2/HNK-1 carbohydrate has been implicated recently in the preferential growth of motor neurons under regenerative conditions in the adult mouse, and it has been proposed that the expression of the L2/HNK-1 carbohydrate in mature myelin sheaths and endoneurial Schwann cell tubes might be a foresighted predisposition for axonal regrowth following a lesion (Y. Xin, B. Schmitz, M. Schachner, and R. Martini, submitted). This observation is in line with the hypothesis that neural carbohydrate structures provide particular sets of neurons with special cues for target selection. However, the exact carbohydrate structures have not been determined for any neural adhesion molecule nor has the L2/HNK-1-reactive epitope been structurally defined on a glycoprotein.

To investigate the nature of the L2/HNK-1 epitope on a purified neural glycoprotein, L2-reactive P_0 was purified on an L2-immobilized affinity column in order to enrich the L2-reactive carbohydrate structure(s). The chemical method hydrazinolysis was used to release the oligosaccharides present on P_0.

MATERIALS AND METHODS

Preparation of P_0

L2-positive P_0 was isolated as described by Bollensen and Schachner (1987). Essentially, a crude membrane preparation (Rathjen and Schachner, 1984) was isolated from human sciatic nerves and extracted with detergent. This extract was centrifuged at 100,000 *g* for 1 h at 4°C and the supernatant was passed sequentially over a column of a monoclonal antibody to myelin-associated glycoprotein and then over a monoclonal L2 antibody column (Kruse et al., 1984), followed by elution of the bound protein. Purification was monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie Blue staining, and western blot analysis.

Isolation and reduction of N-linked oligosaccharides

Purified P_0 was dialysed exhaustively against glass-distilled water (4°C) and cryogenically dried over activated charcoal at −196°C ($<10^{-6}$ bar). Oligosaccharides were released by treatment with fresh double vacuum-distilled anhydrous hydrazine and purified as described previously (Ashford et al., 1987). Oligosaccharides were converted to radiolabelled oligosaccharide alditols by reduction with 6 m*M* NaB^3H_4 (10 Ci/mmol) and 1 m*M* glycan at 30°C in 50 m*M* NaOH buffered to pH 11.0 with saturated boric acid. After 4 h, an equal volume of 1 *M* NaB^2H_4 in buffered 50 m*M* NaOH, pH 11, was added and the reaction continued for an additional 2 h. The oligosaccharides were then purified from the reagents and radiochemical contaminants as described elsewhere (Ashford et al., 1987). Radiolabelled oligosaccharides were separated by high-voltage paper electrophoresis (HVE) in the presence of pyridine–acetate buffer, pH 5.4. Radioactivity was detected using a linear radiochromatographic scanner (Berthold TLC Scanner, Lab Impex, Winnersh, U.K.).

All general reagents used were of analytical grade or higher and were obtained from previously described sources (Ashford et al., 1987; Parekh et al., 1989).

Cleavage of anionic groups of oligosaccharides

Sulphate esters were cleaved by mild methanolysis (Lechner et al., 1985). Oligosaccharides were lyophilised in glass vessels equipped with a reactivalve (Pierce). Then, 500 μl of dry 50 m*M* methanolic-HCl (made by diluting 0.5 *M* anhydrous methanolic-HCl with dry methanol) was introduced into the vessel through the valve, and the reaction allowed to proceed at room temperature for 18 h. The reaction was stopped by removing the solution from the vessel and evaporating to dryness under reduced pressure. Residual HCl was removed by evaporation from 500 μl of water, and the residue was re-*N*-acetylated by the addition of 500 μl of saturated sodium bicarbonate solution and two aliquots, separated by an interval of 10 min, of 20 μl of acetic anhydride (Fluka). The reaction was allowed to proceed at room temperature for 50 min, then the oligosaccharides were desalted by passage through 500 μl of AG50 × 12 (H^+ form) resin, eluted in water, and concentrated by evaporation. Deesterification of any carboxyl group was performed by treating the sample with 50 m*M* NaOH at 50°C for 2 h. The products of the reaction were analysed by HVE as described above.

The standard biantennary oligosaccharide [Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAcOT, where Gal is galactose, GlcNAc is *N*-acetylglucosamine, Man is mannose, and OT refers to the reduced alditol] was also subjected to the desulphation procedure and shown by Bio-Gel P4 filtration analysis not to be degraded. Methods for the preparation of standard oligosaccharides have been described elsewhere (Parekh et al., 1987, 1989). Desialylation was performed by mild acid treatment as described earlier (Green and Baenziger, 1988) or with neuraminidase from *Arthrobacter ureafaciens*.

Bio-Gel P4 gel filtration chromatography

Reduced neutral oligosaccharides were fractionated by gel filtration chromatography using Bio-Gel P4 (−400 mesh; 2 m × 1.5 cm) in water at 200 μl/min, and at 55°C. Typically, the eluate was collected as 200-μl fractions. The hydrodynamic volume of the material that eluted from the Bio-Gel P4 columns was determined by comparison with coinjected isomaltose oligomer oligosaccharides (dextran hydrolysate), as monitored on an ERMA refractometer. The elution positions of the reduced radioactive oligosaccharides were determined by monitoring the eluate from the Bio-Gel P4 column with an HPLC radioactivity monitor (Berthold model LB503, Lab Impex). In addition, the eluate was monitored by withdrawing aliquots from the fractions for liquid scintillation counting.

Glycosidases

Exoglycosidases were used to determine various monosaccharide components, anomers, and linkages. The following enzymes were used: jack bean α-mannosidase, bovine epididymal β-galactosidase, jack bean β-hexosaminidase, *Streptococcus pneumoniae* β-hexosaminidase, bovine epididymal α-fucosidase, and *Bacteriodes fragilis* endo-β-galactosidase. Glycans were grouped into classes (oligomannose, hybrid, complex, polylactosamine, etc.) using the following criteria: oligomannose (original elution positions 8.9 → 12.8, suscep-

tible to jack bean α-mannosidase, and eluting at 5.5 glucose units (g.u.) from Bio-Gel P4 after digestion), hybrid (sensitive to α-mannosidase, losing one or two mannose residues, and sensitive to β-galactosidase and/or β-hexosaminidase following α-mannosidase digestion), complex (resistant to α-mannosidase, sensitive to β-galactosidase and/or β-hexosaminidase), and poly-*N*-acetyllactosamines [containing (Galβ1-4GlcNAcβ3→)$_n$ repeats, sensitive to endo-β-galactosidase from *Bacteriodes fragilis*].

The reduced oligosaccharides (0.01–1 n*M*) were digested with exoglycosidases essentially as described elsewhere (Parekh et al., 1987, 1989) with the following additions: digestion with bovine epididymal β-galactosidase was performed in a reaction volume of 25 μl containing 0.2 U/ml of enzyme in 0.1 *M* citrate–phosphate buffer, pH 6.0. *Streptococcus pneumoniae* β-hexosaminidase digestion was performed in a reaction volume of 20 μl at 0.3 U/ml of enzyme in 0.1 *M* citrate–phosphate buffer, pH 6.0. *Bacteriodes fragilis* endo-β-galactosidase was purchased from Boehringer–Mannheim; all other enzymes were obtained from previously described sources (Parekh et al., 1987, 1989).

TABLE 1. *Molar percentage of P_0 oligosaccharide fractions*

Untreated		Postneuraminidase	
Peak	Percentage	Peak	Percentage
SN	9.6	S'N	18.9
S1	17.3	S'1	42.4
S2	25.0	S'2	17.9
S3	21.8	S'3	20.8
S4	26.4		
S5	minor		
S6	minor		

In the untreated condition, the molar proportions of the major fractions were obtained by integration of the radioelectrophoretogram shown in Fig. 1, top panel. Peaks are listed in order of increasing electrophoretic mobility. In the postneuraminidase condition, the peak percentages were obtained by elution with water of the relevant portion of the radioelectrophoretogram shown in Fig. 1, bottom panel, followed by liquid scintillation counting.

RESULTS

The glycoprotein purified from human sciatic nerve myelin by immunoaffinity chromatography (Bollensen and Schachner, 1987) was shown to be P_0- and L2/HNK-1-immunoreactive by western blot analysis exactly as reported earlier (Bollensen and Schachner, 1987), and was also a single band at 29 kDa by Coomassie-stained SDS-PAGE (as in Bollensen and Schachner, 1987). Approximately 90% of the anti-P_0-reactive material was retained on the L2 column.

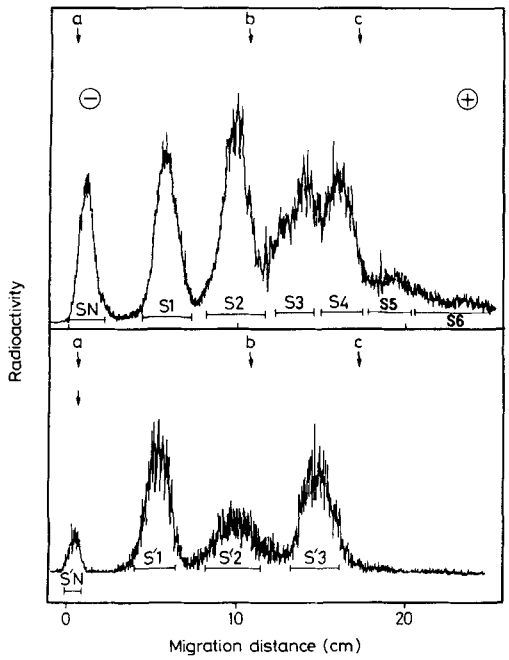

FIG. 1. High-voltage radioelectrophoretogram of L2-positive human P_0 oligosaccharides. **Top panel:** Total L2-reactive P_0 oligosaccharides. The migration positions of [^{3}H]lactitol, [^{3}H]sialyllactitol, and the dye bromophenol blue are indicated by a, b, and c, respectively. Reference to peak labelling is made in the text. **Bottom panel:** Same as top panel, but following mild hydrolysis of the glycans (with acetic acid) to remove sialic acid residues.

When the reduced glycans from human P_0 were analysed by pyridine–acetate HVE, considerable heterogeneity of charge was seen (Fig. 1, top panel) with five major peaks (SN and S1–S4 inclusive) and minor species (S5 and S6) observed. The relative molar percentages of the major fractions are given in Table 1. Treatment of the acidic oligosaccharides with *Arthrobacter ureafaciens* neuraminidase or mild acid resulted in one neutral and three acidic peaks (Fig. 1, bottom panel and Table 1). The three acidic peaks, designated S'1–S'3 were treated separately with 50% aqueous HF to hydrolyse phosphate esters specifically from the glycans (Ferguson et al., 1988). No change in the respective migration positions of the peaks on subsequent HVE was observed, indicating the absence of phosphate esters on the glycans. The same fractions were then subjected to partial desulphation. Figure 2 shows that in addition to the starting material, each fraction generated a neutral peak and a series of charged peaks that migrated intermediate between neutral and the pre-hydrolysis migration positions. When the residual acidic material was subjected to a second methanolysis, essentially all the radioactivity was converted to neutral components. The efficiency of the neutralization is reported in Table 2. From the partial desulphation data it can be concluded that the S'1 peak contains one sulphate residue, S'2 contains two sulphate residues, and S'3 contains three sulphate residues. Fractions S5 and S6 (Fig. 1, top panel) could contain three sulphate esters as well as one or more sialic acid residues. The fraction of the material neutralized by neuraminidase (S'N) contained sialic acid as the only acidic component.

An aliquot of each acidic fraction (S'1, S'2, and S'3) was treated with endoglycosidase H after neuramini-

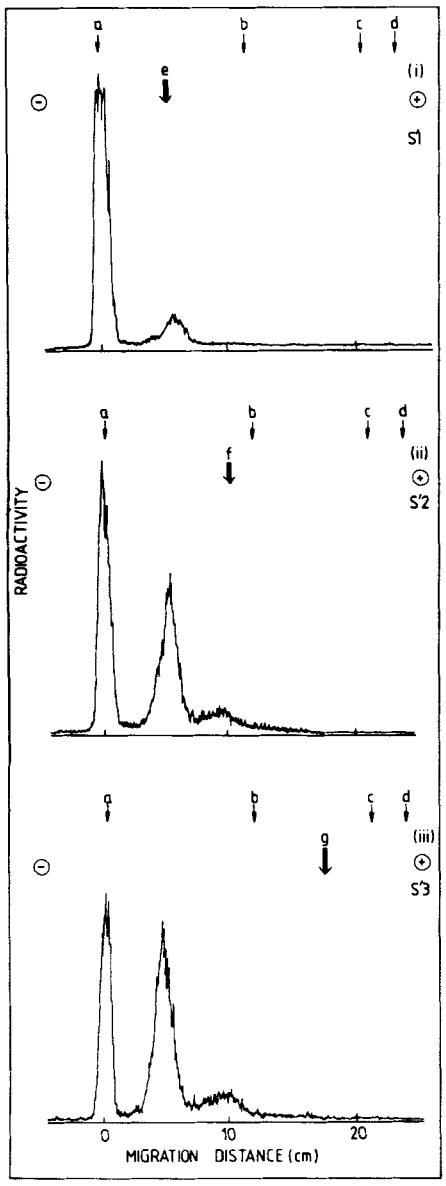

FIG. 2. High-voltage radioelectrophoretogram of fractions S'1, S'2, and S'3 from Fig. 1, bottom panel, after partial methanolysis. The migration positions a, b, and c are as indicated in Fig. 1. In addition, d represents *N*-[^{3}H]acetylglucosaminitol-6-sulphate, and the migration positions for S'1, S'2, and S'3 before partial methanolysis are indicated by the bold arrows e, f, and g, respectively.

dase treatment and the radiolabelled products analysed by HVE. No differences in the migration behaviour of the glycans was observed (data not shown), indicating that these sulphated oligosaccharides were not sensitive to the enzyme.

When the individual HVE fractions S'N, S'1, S'2, and S'3 were neutralized and subjected to Bio-Gel P4 gel filtration chromatography, the elution profiles shown in Fig. 3 (panels a–d) were obtained. From these chromatograms it was apparent that the glycans from L2/HNK-1 expressing human P_0 display a considerable degree of monosaccharide sequence heterogeneity, in addition to the charge heterogeneity detected above by HVE.

To determine the representative monosaccharide sequences present on P_0, fraction N-S'2 was subjected to a series of exoglycosidase digestions. Digestion of pool B of N-S'2 with jack bean α-mannosidase caused no change in profile (Fig. 4a) and did not result in any material eluting at 5.5 g.u. where Manβ4GlcNAcβ4GlcNAc the expected digestion product would have eluted if oligomannose structures had been present. When the α-mannosidase-treated pool was digested with a mixture of jack bean β-galactosidase and jack bean β-hexosaminidase, only two peaks eluting at 7.5 and 6.5 g.u. were seen (Fig. 4b). The 6.5-g.u. structures could have been generated only from structures having an exposed α-mannose on one arm (the α6 arm for hybrid-type structures). The 7.5-g.u. structure could have been generated from complex-type structures or core fucosylated hybrids. The latter was confirmed using the following enzyme digests: Treatment with jack bean α-mannosidase converted the 7.5- and 6.5-g.u. structures to ones eluting at 6.5 and 5.5 g.u., respectively, and treatment of the 6.5-g.u. structure with α-fucosidase resulted in a product eluting at 5.5 g.u. Similar results were found for structures eluting between 11 and 13 g.u. for N-S'1. Also present were structures eluting at 14.5 and 13.5 g.u., which were of the complex class.

Pool A of N-S'2 was treated with endo-β-galactosidase to determine whether these structures contained repeating polylactosamine saccharides. Figure 5 shows that three new structures eluting at 10.3 (IV), 11.3 (III), and 13.5 (II) g.u. were generated by treatment with endo-β-galactosidase. A repeat digest of pool A (I) converted a further 30% of the material to a similar pattern of digestion fragments.

TABLE 2. *Efficiency of neutralization by methanolysis of acidic oligosaccharides from human P_0 (fractions S'1–S'3) after desialylation*

Fraction	First treatment (% neutralized)	Second treatment (% neutralized)
S'1	87	58
S'2	72	42
S'3	65	36

The data were obtained by treatment of S'1–S'3 individually with methanolic HCl, followed by fractionation of the products by HVE, as described in Materials and Methods. The neutral and acidic glycans were eluted separately from the paper, and quantified by liquid scintillation spectroscopy. The acidic material was then subjected to a second methanolic-HCl treatment. If the efficiency of release of a sulphate residue after the first treatment is 87% (i.e., S'1), then S'2 and S'3 should have values of 75 and 66%, respectively, which agrees with the experimentally determined values of 72 and 65%, respectively. The data for the second treatment represent the proportions of the glycans that were not neutralized by the first treatment, but were subsequently neutralized by the second methanolysis.

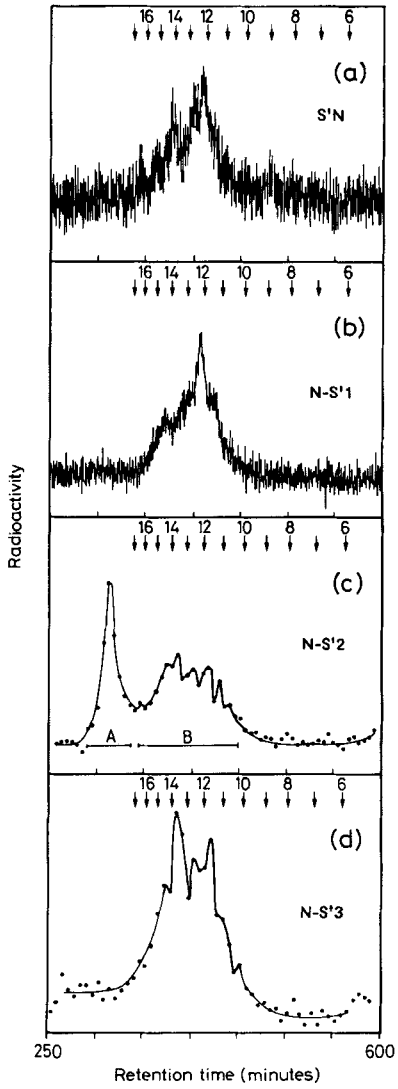

FIG. 3. High-resolution Bio-Gel P4 chromatograms of neutralized P_0 oligosaccharides. Chromatogram for S'N, with oligosaccharides neutralized by desialylation (**a**); chromatogram for N-S'1, with oligosaccharide fraction S'1 neutralized by methanolysis (monosulphated glycans) (**b**); chromatogram for N-S'2, with oligosaccharide fraction S'2 neutralized by methanolysis (disulphated glycans) (**c**); and chromatogram for N-S'3, with oligosaccharide fraction S'3 neutralized by methanolysis (trisulphated glycans) (**d**).

DISCUSSION

The heterogeneity of the glycan structures observed in this study, whether of monosaccharide sequence or anionicity, is not likely to be attributable to multiple glycosylation sites on P_0, as the primary structure of the polypeptide shows remarkable homology between species (bovine, rat, shark), and because it is most likely that the single *N*-glycosylation site observed in these mammalian glycoproteins is conserved in the human protein (Saavedra et al., 1989). Furthermore, the carbohydrate composition of P_0, as found by Roomi et al. (1978), does not support the existence of "O"-linked glycans on the molecule, especially as *N*-acetylgalactosamine (GalNAc) was absent. If the L2 monoclonal antibody recognized a distinct carbohydrate epitope, it is evident that this epitope must reside on a variety of oligosaccharide chains, particularly because 90% of anti-P_0-reactive material was retained on the L2 column in this study. This was similar to the yield of glycopeptides carrying sulphated glycans found by Kitamura et al. (1981). Any additional immunoreactive material that may have been retarded by the L2 column step, including other glycoprotein species, would have resulted in detectable heterogeneity and subsequent elimination during the procedures of SDS-PAGE, western blot analysis, and electroelution (see Bollensen and Schachner, 1987).

These results clearly support sulphation as a major structural element in the carbohydrates of L2/HNK-1-immunoreactive P_0. The exact location of the sulphate residues is intriguing. The presence of core fucose (Fuc) [i.e., R-GlcNAcβ4(Fucα6)GlcNAc] suggests that if one of the sulphates is on the core, then the reducing terminal GlcNAc would have to be disubstituted or the sulphate would have to be on the nonreducing GlcNAc (e.g., as seen by Merkle et al., 1985). Sulphate

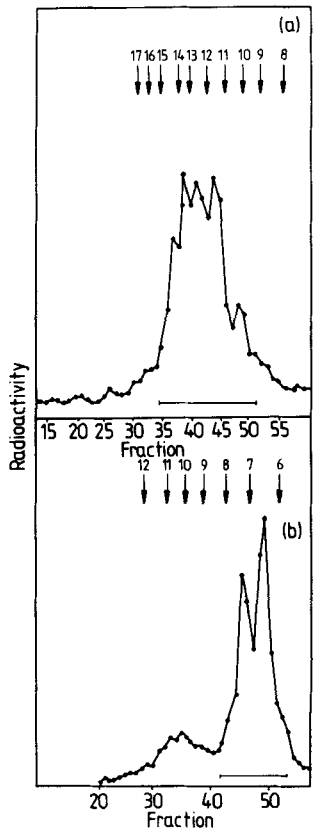

FIG. 4. High-resolution Bio-Gel P4 chromatogram of the oligosaccharides shown eluting in pool B of Fig. 3c following digestion with jack bean α-mannosidase (**a**) and jack bean β-galactosidase and β-hexosaminidase (**b**).

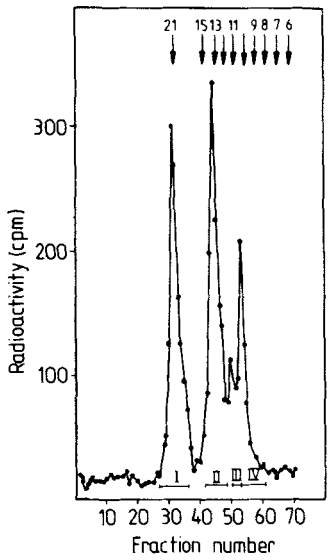

FIG. 5. High-resolution Bio-Gel P4 chromatogram of the oligosaccharides present in pool A of Fig. 3c following digestion with endo-β-galactosidase. The predigestion elution position is shown as fraction I.

has been found in a number of N-linked oligosaccharides from a variety of sources (Roux et al., 1988 and references therein). Man-6-SO_4 (Freeze and Wolgast, 1986), Man-4-SO_4 (Yamashita et al., 1983), GalNAc-4-SO_4 (Green and Baenziger, 1988), and GlcNAc-4-SO_4 and GlcNAc-6-SO_4 (Edge and Spiro, 1984) have been reported as possible structures for sulphation. GlcNAc-4-SO_4 would most likely occur on the nonreducing terminus of N-linked oligosaccharides, whereas GlcNAc-6-SO_4 can occur in either reducing or nonreducing positions (Edge and Spiro, 1984). The presence of both sialic acid and sulphate on a single structure has also been reported in mammalian cells (Roux et al., 1988).

Kitamura et al. (1981) previously have described five different species of carbohydrate on P_0 as being neutral, monosialylated, monosulphated, both monosialylated and monosulphated, and disulphated by the analysis of glycopeptides from diethylaminoethyl-Sephadex A25 chromatography. Our results indicate further that there are species of P_0 that contain up to three sulphate residues. A large proportion of the glycans appear to be endoglycosidase H-resistant nonbisected hybrid class oligosaccharides. The resistance to endoglycosidase H could have resulted from sulphation of the reducing core (Freeze et al., 1983), the presence of core fucose (Ivatt et al., 1984), or truncation of the oligosaccharide arm bearing the nonreducing terminal mannose (6 arm) (Maley et al., 1989). The latter is a common characteristic of CNS glycoprotein glycans (Thomas et al., 1988). The carbohydrate composition of the P_0 glycopeptide fractions reported by Kitamura et al. (1981) was also consistent with hybrid class oligosaccharide structures rather than with complex-type structures (i.e., Man/Gal ratio = 4), of which the hybrid oligosaccharide Galβ4GlcNAcβ2Manα3(Manα3Manα6)-Manβ4GlcNAcβ4(Fucα6)GlcNAc was believed to be a common constituent. This structure would elute at ~12.2 g.u. on Bio-Gel P4 chromatography, and fractions of this size are readily seen in all panels of Fig. 3. The exoglycosidase digestions shown in Fig. 4a and b are compatible with the structure, although it is clear from Fig. 3 that other oligosaccharides are also present.

The L2/HNK-1 epitope of glycoproteins has been inferred to include a sulphated glucuronic acid essentially because L2/HNK-1 monoclonal antibodies will show reactivity against certain glycoproteins (Mikol et al., 1988; Yamamoto et al., 1988; Gowda et al., 1989; Burger et al., 1990), but direct structural proof that the glycoprotein epitope is identical to that established for glycolipids is lacking. Thus Shashoua et al. (1986) did not directly demonstrate the presence of sulphate on glucuronic acid derived from HNK-1-positive glycoprotein, and the work of Schwarting et al. (1987) also is not unequivocal. Indeed, it has been suggested that the HNK-1 carbohydrate epitope on the myelin-associated glycoprotein is not necessarily sulphated glucuronic acid (Quarles, 1989). In accordance with this, no evidence was found in the present study for such a structure in the L2-reactive glycans of P_0. Overall, we were able to neutralize >95% of the oligosaccharide from L2-positive P_0 molecules using neuraminidase and methanolysis, which rules out the presence of glucuronic acid in direct glycosidic linkage on the majority of the L2-positive P_0 oligosaccharides. However, we cannot exclude that the glucuronic acid is neutral, due to lactone formation. The ability to digest sequentially the bulk of the oligosaccharides with conventional exoglycosidases excludes potential "stop points" in the sequence (i.e., no glucuronidase was necessary for complete digestion of the oligosaccharide chains). Further studies are required to test whether a sulphate-3'-glucuronic acid residue could be attached via a sulphate diester linkage to one or more of the other sulphate residues rather than be in glycosidic linkage to the carbohydrate chain. By analogy, Man-6-phosphate residues of phosphorylated oligomannose and hybrid structures can be found in diester linkage (e.g., Freeze et al., 1983; Gabel et al., 1984).

The P_0 glycoforms, identified in this study as L2-reactive, contained up to 10% neutral glycans (Table 1), which is not consistent with the view that the L2 carbohydrate epitope is sulphated. It is known, however, that P_0 can interact homophilically with itself (Filbin et al., 1990; Schneider-Schaulies et al., 1990), so it is possible that this 10% fraction could reflect L2-negative P_0 that bound to L2-positive P_0 on the column. This proportion of potential L2-negative glycans would be insufficient to mask significantly the nature of the oligosaccharides of the L2-positive preparation. A similar percentage of total P_0 was definitively shown to be L2-negative from its lack of retention on the L2 column (see above). Isolation and characterization of the L2 carbohydrate epitope by direct immunoaffinity chro-

matography of the oligosaccharides has not been possible as free carbohydrates exhibit a lower affinity to antibodies than those bound to protein or lipid.

The function of P_0 glycoforms expressing the heterogeneity observed in this study has not yet been defined. It is known from recombinant studies with P_0 (Schneider-Schaulies et al., 1990), however, that only one glycosylated partner is essential for heterophilic or homophilic interactions involving P_0. The heterophilic interaction shown with neurons (Schneider-Schaulies et al., 1990) may be partly responsible for the recognition between axon and the myelinating Schwann cell at the onset of myelination, whereas the homophilic interaction could be indicative of the role of P_0 in the self-recognition of apposing loops of Schwann cell surface membrane during the myelination process and of the mature compact myelin sheath (Trapp, 1988; Schneider-Schaulies et al., 1990).

Because carbohydrate epitopes such as L2/HNK-1 and L3 also occur on other adhesion molecules and cell types (Kruse et al., 1984, 1985; Rathjen and Schachner, 1984; Martini and Schachner, 1986; Noronha et al., 1986; Poltorak et al., 1987), they may be the key structures in the heterophilic interactions. This hypothesis is supported in the case of P_0 by the incorporation of sulphate into its glycans at the onset of myelination (Poduslo, 1989). The detection in the present study of multisulphated species of N-linked glycans on P_0 suggests that the degree of glycan sulphation will be critical for the nature of the heterophilic and homophilic interactions, and perhaps not necessarily solely for the promotion of adhesive processes.

Acknowledgment: We are grateful to Marilyn Tasker for preparation of the manuscript. The Oxford Glycobiology Institute is supported by the Monsanto Company. B.S., E.B., and M.S. are supported by the Deutsche Forschungsgemeinschaft (SFB 317).

REFERENCES

Ariga T., Kohriyama T., Freddo L., Latov N., Saito M., Kon K., Ando S., Suzuki M., Hemling M. E., Rinehart K. L., Kusunoki S., and Yu R. K. (1987) Characterisation of sulfated glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. *J. Biol. Chem.* **262,** 848–853.

Ashford D. A., Dwek R. A., Welply J. K., Amatayakul S., Homans S. W., Lis H., Taylor G. N., Sharon N., and Rademacher T. W. (1987) The β1→2-D-xylose and α1→3-L-fucose substituted N-linked oligosaccharides from *Erythrina cristagalli* lectin: isolation, characterisation, and comparison with other legume lectins. *Eur. J. Biochem.* **166,** 311–320.

Bollensen E. and Schachner M. (1987) The peripheral myelin glycoprotein P_0 expresses the L2/HNK-1 and L3 carbohydrate structures shared by neural adhesion molecules. *Neurosci. Lett.* **82,** 77–82.

Burger D., Simon M., Perruisseau G., and Steck A. J. (1990) The epitope(s) recognized by HNK-1 antibody and IgM paraprotein in homopathy is present on several N-linked oligosaccharide structures on human P_0 and myelin-associated glycoprotein. *J. Neurochem.* **54,** 1569–1575.

Chou D. K. and Jungalwala F. B. (1988) Sulfoglucuronyl neolactoglycolipids in adult cerebellum: specific absence in murine mutants with Purkinje cell abnormality. *J. Neurochem.* **50,** 1655–1658.

Chou D. K. H., Ilyas A. A., Evans J. E., Quarles R. H., and Jungalwala F. B. (1985) Structure of a glycolipid reacting with monoclonal IgM in neuropathy and with HNK-1. *Biochem. Biophys. Res. Commun.* **128,** 383–388.

Chou D. K. H., Ilyas A. A., Evans J. E., Costello C., Quarles R. H., and Jungalwala F. B. (1986) Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. *J. Biol. Chem.* **261,** 11717–11725.

Chou D. K. H., Schwarting G. A., Evans J. E., and Jungalwala F. B. (1987) Sulfoglucuronyl-neolacto series of glycolipids in peripheral nerves reacting with HNK-1 antibody. *J. Neurochem.* **49,** 865–873.

Edge A. S. B. and Spiro R. G. (1984) Presence of sulfate in N-glycosidically linked carbohydrate units of calf thyroid plasma membrane glycoproteins. *J. Biol. Chem.* **259,** 4710–4713.

Ferguson M. A. J., Homans S. W., Dwek R. A., and Rademacher T. W. (1988) Glycosylphosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science* **239,** 753–759.

Filbin M. T., Walsh F. S., Trapp B. D., Pizzey J. A., and Tennekoon G. I. (1990) Role of myelin P_0 protein as a homophilic adhesion molecule. *Nature* **344,** 871–872.

Freeze H. H. and Wolgast D. (1986) Structural analysis of N-linked oligosaccharides from glycoproteins secreted by *Dictyostelium discoideum*. *J. Biol. Chem.* **261,** 127–134.

Freeze H. H., Yeh R., Miller A. L., and Kornfeld S. (1983) Structural analysis of the asparagine-linked oligosaccharides from three lysosomal enzymes of *Dictyostelium discoideum*: evidence for an unusual acid-stable phosphodiester. *J. Biol. Chem.* **258,** 14874–14879.

Gabel C. A., Costello C. E., Reinhold V. N., Kurz L., and Kornfeld S. (1984) Identification of methylphosphomannosyl residues as components of the high mannose oligosaccharides of *Dictyostelium discoideum* glycoproteins. *J. Biol. Chem.* **259,** 13762–13769.

Gowda D. C., Margolis R. U., and Margolis R. K. (1989) Presence of HNK-1 epitope on poly(*N*-acetyllactosaminyl) oligosaccharides and identification of multiple core proteins in the chondroitin sulfate proteoglycans of brain. *Biochemistry* **28,** 4468–4474.

Green E. D. and Baenziger J. U. (1988) Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *J. Biol. Chem.* **263,** 25–35.

Greenfield S., Brostoff S., Eylar E. H., and Morell P. (1973) Protein composition of myelin in the peripheral nervous system. *J. Neurochem.* **20,** 1207–1216.

Ivatt R. L., Das O. P., Henderson E. J., and Robbins P. W. (1984) Glycoprotein biosynthesis in *Dictyostelium discoideum*: developmental regulation of the protein-linked glycans. *Cell* **38,** 561–567.

Kitamura K., Sakamoto Y., Suzuki M., and Uyemura K. (1981) Microheterogeneity of carbohydrate in P_0 protein from bovine peripheral nerve myelin, in *Glycoconjugates: Proceedings of the 6th International Symposium on Glycoconjugates, Tokyo.* (Yamakawa T., Osawa T., and Handa S., eds), pp. 273–274. Japan Scientific Press, Tokyo.

Kruse J., Mailhammer R., Wernecke H., Faissner A., Sommer I., Goridis C., and Schachner M. (1984) Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature* **311,** 153–155.

Kruse J., Keilhauer G., Faissner A., Timpl R., and Schachner M. (1985) The J1 glycoprotein: a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature* **316,** 146–148.

Kunemund V., Jungalwala F. B., Fischer G., Chou D. K. H., Keilhauer G., and Schachner M. (1988) The L2/HNK-1 carbohydrate

of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* **106,** 213–223.

Lechner J., Wieland F., and Sumper M. (1985) Biosynthesis of sulfated saccharides N-glycosidically linked to the protein via glucose. *J. Biol. Chem.* **260,** 860–866.

Lemke G. and Axel R. (1985) Isolation and sequence of a cDNA encoding the major structural protein of peripheral myelin. *Cell* **46,** 501–508.

Maley F., Trimble R. B., Tarentino A. L., and Plummer T. H. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal. Biochem.* **180,** 195–204.

Martini R. and Schachner M. (1986) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, MAG) and their shared carbohydrate epitope and myelin basic protein (MBP) in developing sciatic nerve. *J. Cell Biol.* **103,** 2439–2448.

Martini R., Bollensen E., and Schachner M. (1988) Immunocytological localization of the major peripheral nervous system glycoprotein P_0 and its L2/HNK-1 and L3 carbohydrate structure in developing and adult mouse sciatic nerve. *Dev. Biol.* **129,** 330–338.

Merkle R. K., Elbein A. D., and Heifetz A. (1985) The effect of swainsonine and castanospermine on the sulfation of the oligosaccharide chains of N-linked glycoproteins. *J. Biol. Chem.* **260,** 1083–1089.

Mikol D. D., Wrabetz L., Marton L. S., and Stefansson K. (1988) Developmental changes in the molecular weights of polypeptides in the human CNS that carry the HNK-1 epitope and bind *Phaseolus vulgaris* lectins. *J. Neurochem.* **50,** 1924–1928.

Noronha A., Ilyas A., Quarles R. H., Antonicek H., and Schachner M. (1986) Molecular specificity of L2 monoclonal antibodies that bind to carbohydrate determinants of neural cell adhesion molecules and resemble other monoclonal antibodies recognizing the myelin-associated glycoprotein. *Brain Res.* **385,** 237–244.

Parekh R. B., Tse A. G. D., Dwek R. A., Williams A. F., and Rademacher T. W. (1987) Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1. *EMBO J.* **6,** 1233–1244.

Parekh R. B., Dwek R. A., Thomas J. R., Opdenakker G., Rademacher T. W., Wittwer A. J., Howard S. C., Nelson R., Siegel N. R., Jennings M. G., Harakas N. K., and Feder J. (1989) Cell-type-specific and site-specific N-glycosylation of type I and type II human tissue plasminogen activator. *Biochemistry* **28,** 7644–7662.

Poduslo J. F. (1989) Golgi sulfation of the oligosaccharide chain of P_0 occurs in the presence of myelin assembly but not in its absence. *J. Biol. Chem.* **265,** 3719–3725.

Poltorak M., Sadoul R., Keilhauer G., Landa C., Fahrig T., and Schachner M. (1987) Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron–oligodendrocyte and oligodendrocyte–oligodendrocyte interaction. *J. Cell Biol.* **105,** 1893–1899.

Quarles R. H. (1989) Glycoproteins of myelin and myelin-forming cells, in *Neurobiology of Glycoconjugates* (Margolis R. U. and Margolis R. K., eds), pp. 243–275. Plenum Press, New York.

Rathjen F. G. and Schachner M. (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J.* **3,** 1–10.

Roomi M. W., Ishaque A., Khan N. R., and Eylar E. H. (1978) The P_0 protein: the major glycoprotein of peripheral nerve myelin. *Biochim. Biophys. Acta* **536,** 112–121.

Roux L., Holojda S., Sundblad G., Freeze H. H., and Varki A. (1988) Sulfated N-linked oligosaccharides in mammalian cells. *J. Biol. Chem.* **263,** 8879–8889.

Saavedra R. A., Fors L., Aebersold R. H., Arden B., Horvath S., Sanders J., and Hood L. (1989) The myelin proteins of the shark brain are similar to the myelin proteins of the mammalian peripheral nervous system. *J. Mol. Evol.* **29,** 149–156.

Sakamoto Y., Kitamura K., Yoshimura K., Nishijima T., and Uyemura K. (1987) Complete amino acid sequence of P_0 protein in bovine peripheral nerve myelin. *J. Biol. Chem.* **262,** 4208–4214.

Schneider-Schaulies J., von Brunn A., and Schachner M. (1990) Recombinant peripheral myelin protein P_0 confers both adhesion and neurite outgrowth promoting properties. *J. Neurosci. Res.* **27,** 286–297.

Schwarting G. A., Jungalwala F. B., Chou D. K. H., Boyer A. M., and Yamamoto M. (1987) Glucuronic acid- and sulfate-containing glycoconjugates are temporally and spatially regulated antigens in the developing mammalian central nervous system. *Dev. Biol.* **120,** 65–76.

Shashoua V. E., Daniel P. F., Moore M. E., and Jungalwala F. B. (1986) Demonstration of glucuronic acid on brain glycoproteins which react with HNK-1 antibody. *Biochem. Biophys. Res. Commun.* **138,** 902–909.

Thomas J. R., Parekh R. B., Wing D. R., Dwek R. A., Rademacher T. W., Thomas-Oates J. E., Dell A., and Schachner M. (1988) Structural characterization of exoglycosidase-resistant N-linked oligosaccharides from mammalian brain glycoproteins. *Proceedings of the 14th International Carbohydrate Symposium (Stockholm)* Abs. No. A25.

Trapp B. D. (1988) Distribution of the myelin-associated glycoprotein and P_0 protein during myelin compaction in quaking mouse peripheral nerve. *J. Cell Biol.* **107,** 675–685.

Yamamoto M., Marshall P., Hemmendinger L. M., Boyer A. B., and Caviness V. S. (1988) Distribution of glucuronic acid- and sulfate-containing glycoproteins in the central nervous system of the adult mouse. *Neurosci. Res.* **5,** 273–298.

Yamashita K., Ueda I., and Kobata A. (1983) Sulfated asparagine-linked sugar chains of hen egg albumin. *J. Biol. Chem.* **258,** 14144–14147.