The carbohydrate structures present on the glycoproteins in the central and peripheral nerve systems are essential in many cell adhesion processes. The P0 glycoprotein, expressed by myelinating Schwann cells, plays an important role during the formation and maintenance of myelin, and it is the most abundant constituent of myelin. Using monoclonal antibodies, the homophilic binding of the P0 glycoprotein was shown to be mediated via the human natural killer cell (HNK)-1 epitope (3-O-SO₂H-GlcUA(1→3)Gal(1→4)GlcNAc) present on the N-glycans. We recently described the structure of the N-glycan carrying the HNK-1 epitope, present on bovine peripheral myelin P0 (Voshol, H., van Zuylen, C. W. E. M., Orberger, G., Vliegenthart, J. F. G., and Schachner, M. (1996) J. Biol. Chem. 271, 22957–22960). In this study, we report on the structural characterization of the carbohydrate moieties present on the single N-glycosylation site, using state-of-the-art NMR and mass spectrometry techniques. Even though all structures belong to the hybrid- or biantennary complex-type structures, the variety of epitopes is remarkable. In addition to the 3-O-sulfate present on the HNK-1-carrying structures, most of the glycans contain a 6-O-sulfated N-acetylgalactosamine residue. This indicates the activity of a 6-O-sulfoglucosamine transferase, which has not been described before in peripheral nervous tissue. The presence of the disialo-, triantennary, and 6-O-sulfosialyl-Lewis X epitopes provides evidence for glycosyltransferase activities not detected until now. The finding of such an epitope diversity triggers questions related to their function and whether events, previously attributed merely to the HNK-1 epitope, could be mediated by the structures described here.

The P0 glycoprotein consists of a single immunoglobulin-like domain in its extracellular part, a transmembranous domain, and a cytoplasmic tail. It is the most abundant protein constituent of peripheral myelin. P0 contains a single N-glycosylation site and heterogeneity in its glycosylation pattern that originates from variable contents of fucose, galactose, and sialic acid residues; sulfate; and the HNK-1 carbohydrate epitope (1→4). P0 appears at the initial stage of myelination and contributes to the formation and maintenance of myelin compaction as an adhesion molecule (5). The essential functional role of P0 in the processes of myelination has been demonstrated by creating P0 knockout mice, which show severe hypomyelination and myelin degeneration (5, 6). In humans, several neurological disorders such as Charcot-Marie-Tooth disease, Dejerine-Sottas disease, and congenital hypomyelination have been associated with mutations in the P0 gene (7).

It has been reported that the glycan moiety of P0 plays an important role in cell-cell adhesion via homophilic binding. This homophilic binding has been mapped to the SDNGT sequence composing amino acids 91–95, which harbors the single N-glycosylation site on P0 (8). Thus, it was observed that this glycopeptide fragment inhibits cell adhesion to a greater extent than the corresponding peptide without glycan (9). Non-glycosylated P0, produced by site-directed mutagenesis, does not show homophilic adhesion (10). In addition, amino acids 43–50 and 74–82 may also contribute to the homophilic binding in a non-carbohydrate-dependent manner (11). Occurrence of age-dependent alteration in the glycan moiety of P0 in the peripheral nerve (12) and mammalian spinal cord (5, 6, 13) has been reported, suggesting that the glycan heterogeneity might be regulated by alterations in physiological conditions, although details of the structural changes of the carbohydrate chain have not yet elucidated.

So far, attempts to identify the oligosaccharides on the P0 glycoprotein using mass spectrometry (2, 14) or various chromatographic techniques (3, 15, 16) have not provided conclusive data concerning the structures of these carbohydrates. Only recently have we described the detailed structural analysis of a major HNK-1-reactive oligosaccharide of the bovine peripheral myelin P0 glycoprotein (17).

In this study, a detailed structural analysis of the carbohydrates contained in the bovine P0 glycoprotein has been performed. The core structure of the carbohydrates and several epitopes, including HNK-1 (17) and 6-O-sulfosialyl-Lewis X (18), were identified using highly sensitive techniques for carbohydrate analysis such as high resolution magic angle spin-
Characterization of N-Glycans of Bovine Peripheral Myelin P0

Sialidase Treatment—Sialidase (Clostridium perfringens, Oxford Glycosciences) digestion was performed by incubating lyophilized ma-
FIG. 2. One-dimensional $^1$H NMR spectra at 500 MHz of the bovine peripheral glycoprotein P0 glycans. Spectra are given with their corresponding fraction numbers, and assignments correspond to the numbering of the residues, depicted in Fig. 1. QN.1–3, Q1.4–6, Q2.9, and Q2.10 are at 285 K (5-mm probe); Q1.7 is at 300 K (nano probe). In the spectrum of QN.3, the 6a and 6b notations correspond to Gal-6 with and without Gal-7, respectively. Peaks marked with asterisks did not originate from carbohydrate material as observed in the two-dimensional NMR spectra. The acetone and acetate peaks are set at 2.225 and 1.908 ppm, respectively. The HDO peak is at 4.92 ppm for 285 K and at 4.77 ppm at 300 K. NAc, N-acetyl; NGc, N-glycolyl.
material in 20 μl of 50 mM sodium acetate buffer (pH 5.0) with 20 milliunits of enzyme for 24 h at 37 °C. Subsequently, the reaction mixture was applied to a Millipore MC membrane filter (5000 nmwl (nominal molecular weight limit), 0.2 cm²) and centrifuged at 15,000 rpm for 15 min. The effluent was lyophilized and permethylated as described below.

Permethylalation—After sialidase digestion, the fractions QN.1 and
Characterization of N-Glycans of Bovine Peripheral Myelin P0

QN.2 were lyophilized and dried over diethylphosphor pentoxide prior to permethylation. Permethylation was performed essentially as described by Ciucanu and Kerek (26). After quenching the reaction, the mixture was washed three times with 700 μl of CHCl₃. The combined CHCl₃ fractions were washed three times with 1 ml of H₂O, dried under a stream of nitrogen, and dissolved in 10 μl of CHCl₃. For MALDI-TOF analysis, 0.5 μl was used.

**Mass Spectrometry—**Negative-ion mode ESI mass spectrometric analyses of fractions Q1.5 and Q2.10 were performed on an Esquire-LC quadrupole ion-trap spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Samples were dissolved in 50 μl of deionized water/acetonitrile (1:1) and were introduced by infusion at a flow rate of 1 μl/min. Ions were scanned (scan range of 50–2100 Da) with a scan speed of 13,000 Da/s at unit resolution using resonance ejection at the hexapole resonance of one-third of the radio frequency (781,250 Hz). The calibration of the mass spectrometer was performed using ESI tuning mixture (Hewlett-Packard Co., Palo Alto, CA). Collision-induced dissociation (CID) tandem mass spectrometric experiments were performed using the quadrupole ion trap to select the precursor ion for fragmentation. Helium was used as collision gas, and the fragmentation energy applied on the end caps varied between 0.5 and 1.8 V. Recorded data were processed using Esquire-NT Version 3.1 software (Bruker Daltonik GmbH). Negative-ion mode mass spectrometric analyses of all other fractions were performed on a Reflex III MALDI-TOF spectrometer (Bruker Daltonik GmbH) equipped with a SCOUT ion source. A 2-GHz digitizer was used. The high mass region of 3000 Da in the reflectron mode using pulsed laser extraction and an acceleration voltage of 25 kV. Aliquots (0.5 μl) of fractions QN.1 and QN.2 were spotted on the target for desalting and permethylation and were deposited on microcrystalline α-cyano-4-hydroxycinnamic acid surfaces and allowed to dry at ambient temperature. The α-cyano-4-hydroxycinnamic acid thin-layer preparation was performed as described by Vorm and Mann (27). The remaining fractions were dissolved in water, diluted 1:10 with trihydroxyacetophenone (17 g/l) of CHCl₃. For further fractionation by high pH anion-exchange chromatography on Mono-Q: QN, Q1 (45 ± 5% of the total), and Q2 (40 ± 5% of the total), eluting at starting buffer without NaCl, starting buffer with a gradient of NaCl from 30 to 100 mM, and starting buffer with a gradient of NaCl from 100 to 400 mM, respectively. Since the Mono-Q pattern varied slightly from run to run, due to the remaining SDS in the mixture, we did not attempt to obtain a better resolution at this stage. Further fractionation by high pH anion-exchange chromatography (19) resulted in three main fractions for QN (QN.1–3), four main fractions for Q1 (Q1.4–7), and three main fractions for Q2 (Q2.8–10) (data not shown). Due to variations in the pulsed amperometric detection response, no estimation of the percentages for the individual fractions could be made. These combined fractions accounted for ~90% of all glycans on P0. At this stage of purification, only three fractions were homogeneous according to one-dimensional ¹H NMR spectroscopy and mass spectrometry; nevertheless, no further purification was carried out in order to prevent any loss of material. Instead, the fractions were analyzed by a combination of mass spectrometry and state-of-the-art NMR spectroscopy. A description is given below of the characteristic features of each fraction, and the deduced structures are summarized in Fig. 1.

**Results**

The carbohydrate pool released from P0 was fractionated into three fractions by anion-exchange chromatography on Mono-Q: QN (15 ± 5% of the total), Q1 (45 ± 5% of the total), and Q2 (40 ± 5% of the total), eluting at starting buffer without NaCl, starting buffer with a gradient of NaCl from 30 to 100 mM, and starting buffer with a gradient of NaCl from 100 to 400 mM, respectively. Since the Mono-Q pattern varied slightly from run to run, due to the remaining SDS in the mixture, we did not attempt to obtain a better resolution at this stage. Further fractionation by high pH anion-exchange chromatography (19) resulted in three main fractions for QN (QN.1–3), four main fractions for Q1 (Q1.4–7), and three main fractions for Q2 (Q2.8–10) (data not shown). Due to variations in the pulsed amperometric detection response, no estimation of the percentages for the individual fractions could be made. These combined fractions accounted for ~90% of all glycans on P0. At this stage of purification, only three fractions were homogeneous according to one-dimensional ¹H NMR spectroscopy and mass spectrometry; nevertheless, no further purification was carried out in order to prevent any loss of material. Instead, the fractions were analyzed by a combination of mass spectrometry and state-of-the-art NMR spectroscopy. A description is given below of the characteristic features of each fraction, and the deduced structures are summarized in Fig. 1.

**QN.1 and QN.2**—The negative-ion mode MALDI-TOF mass spectra of QN.1 and QN.2 (data not shown) after salidase treatment and permethylation showed two peaks at m/z 2095.0 and 2346.2 corresponding to permethylated Hex₃-dHex-HexNAc₂ [M–H]⁻ and Hex₂-dHex-HexNAc₂ [M–H]⁻, respectively, and several other pseudo-molecular ions corresponding to the same structures, but with different degrees of methylation. The one-dimensional ¹H NMR spectra of QN.1 and QN.2 (Fig. 2) revealed the structural reporter group signals (Table I) of a monosialylated hybrid-type structure as described previously (28). For QN.1, two methyl doublets were observed at δ 1.207 and 1.220 (Table I) belonging to the fucose residue α1,6-linked to the Asn-linked GlcNAc-1. The core fucosylation resulted in a downfield shift (~0.043 ppm) of the GlcNAc-2 H-1, which was linked to the GlcNAc-1 α-anomer. The two methyl doublets indicated the presence or absence of a Man-A residue, respectively (29). The observation of two GlcNAc-2 N-acetylsinglets at δ 2.096 and 2.078 (Fig. 2, QN.1) corroborated this finding. These structural features were also observed in most of the other heterogeneous fractions, confirming the structures present in those fractions (Fig. 1) to the hybrid-type category. In the case of QN.2, the main constituent was a hybrid-type structure containing N-glycolylneuraminic acid, as evidenced by the presence of the N-glycolyl singlet at δ 4.116. In addition, the low intensity signal for N-acetyl at δ 2.028 (Fig. 2, QN.2) indicated that fraction QN.2 was contaminated with fraction QN.1. Also in this fraction, part of the structures carried the Man-A residue. QN.—The MALDI-TOF mass spectrum of QN.3 (Fig. 3) in the negative-ion mode revealed four pseudo-molecular ions at m/z 1500.7, 1662.7, 1824.7, and 1986.7 corresponding to Hex₄-dHex-HexNAc₆(SO₃⁻) [M–H]⁻ and the Hex₅Hex, HexN, and Hex₇ analogs, respectively. The structures containing Hex₅, HexN, and Hex₇ were the minor constituents of this fraction, and the latter structure indicated the existence of a terminal Hex-Hex sequence. The one-dimensional ¹H NMR spectrum of QN.3 (Fig. 2) showed the reporter group signals of an asialo hybrid-type structure (30), and the observed chemical shifts are summarized in Table I. The observation of the downfield shift of the GlcNAc-5 H-6a and H-6b resonances to δ 4.405 and 4.348 (Fig. 2, QN.3) revealed the sulfation of this residue as observed by De Waard et al. (31). Two additional α-anomeric signals were observed with respect to QN.1 in the NMR spectrum recorded at 300 K (data not shown) at δ 4.912 and 5.146. The resonance at δ 4.912 corresponded to H-1 of Man-B (28), and the signal at δ 5.146 originated from H-1 of the Hex(a1,6)Gal structure (32, 33). In the TOCSY spectrum (Fig. 4, QN.3), the α-anomeric spin system at δ 5.146 displayed a typical set of three cross-peaks of a Gal residue (Gal-7). Two anomeric resonances were observed for Gal-6 at δ 4.600 and 4.542. The set of cross-peaks on the anomeric track at δ 4.600 revealed the substitution at OH-3 of this Gal-6 with Gal-7 (32). However, the three cross-peaks observed on the anomeric track of Gal-6 at δ 4.542 clearly indicated that this residue was not substituted (32). In summary, fraction QN.3 consists of four hybrid-type structures differing in mass. The Hex₅- and Hex₇-containing structures represented a single compound, whereas the other two could each be present in different glycoforms (Fig. 1).
\[ ^1H \text{ NMR parameters of the P0 glycoprotein oligosaccharides} \]

Table I

| Reporter group | Residue | Q1.1* | QN.2 | QN.3* | Q1.4 | Q1.5 | Q1.6 | Q1.7 | Q2.10 |
|----------------|---------|-------|------|-------|------|------|------|------|-------|
| H-1 GlcNAc-1/β | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| GlcNAc-2 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Man-3 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Man-4 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Man-5 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Man-6 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Man-7 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| GlcNAc-5 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Gal-6 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Gal-7 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Fuc-1 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Fuc-2 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Fuc-3 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Fuc-4 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |
| Fuc-5 | 3.89 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 | 4.31 |

* Double values for the N-acetyl of GlcNAc-2 due to the absence and presence of Man-A.

b Double values for Gal-6 due to the absence and presence of Gal-7.

c Multiple values due to the presence of several structures in the fraction (see Fig. 1).

d NGe, N-glycolyl; NAc, N-acetyl.
the α-anomeric proton at δ 5.112 in combination with the CH₃ doublet at δ 1.166 evidenced the presence of α1,3-fucosylation of GlcNAc-5 (34). Moreover, the observation of the downfield-shifted resonances of the GlcNAc-5 H-6a and H-6b atoms, as in QN.3, demonstrated the 6-O-sulfation of this residue (31). The combined data pointed toward the presence of a 6-O-sulfosialyl-Lewis X structure in this fraction (Fig. 2). The downfield shift of H-3 on the TOCSY track of Gal-6 H-1 (Fig. 4, Q1.4) indicated that Neu5Ac-7 was α2,3-linked to Gal-6. The two-dimensional NOESY NMR spectrum (data not shown) recorded using nano probe MAS NMR spectroscopy, showed NOEs, between Fuc-5 H-1 and Gal-6 H-1 and between Gal-6 H-1 and GlcNAc-5 H-2/3/4, providing evidence for the existence of this structure.

Q1.5 and Q1.6—The negative-ion mode ESI mass spectrum of fraction Q1.5 (Fig. 3) showed two pseudo-molecular ions at m/z 2082.81 and 2104.77 ([M − H]⁻ and [M + Na − 2H]⁻, respectively) belonging to Neu5Ac-Hex₃-dHex-HexNAc(OSO₃). The one-dimensional ¹H NMR spectrum of this structure (Fig. 2) showed reporter group signals as observed for fraction Q1.5 (Table I) with the following differences. An additional N-acetyl signal at δ 2.064 and additional H-3e (δ 2.630) and H-3a (δ 1.738) peaks revealed the presence of two Neu5Ac residues, supporting the mass spectrometric observation and demonstrating the presence of the Neu5Ac(α2-8)Neu5Ac(α2-3)Gal structure (35). The presence of signals at δ 4.117 and 4.139 stemming from Neu5Ac-7 H-8 and H-9, respectively, as observed previously for Neu5Ac(α2-8)Neu5Ac(α2-3)-containing structures (36), confirmed the aforementioned structure. Since no H-3e signal was observed at δ 1.666 (37), the presence of a Neu5Ac(α2-8)Neu5Ac(α2-6)Gal structure could be excluded.

Q2.8—Fraction Q2.8 was described extensively by Voshol et al. (17). Interestingly, this fraction contained only one structure, which carried the HNK-1 epitope at the Man-4′ branch, as evidenced by the NMR data (Table I).
FIG. 4. Two-dimensional $^1$H TOCSY spectra of the carbohydrate structures of QN.3, Q1.4, and Q2.10. Parts of TOCSY spectra recorded at 500 MHz are shown. The spectra of QN.3 and Q1.4 were recorded at 300 K with the MAS NMR nano probe. The spectrum of Q2.10 was recorded at 285 K with the normal probe. For comparison, the structures and the numbering of residues are given in Fig. 1. In the spectrum of QN.3, the 6a and 6b notations correspond to Gal-6 with and without Gal-7, respectively.
Characterization of N-Glycans of Bovine Peripheral Myelin P0

In this study, we describe the detailed structural characterization of the N-linked glycans present on bovine peripheral myelin P0 using state-of-the-art NMR and mass spectrometry techniques. These glycans occur in addition to the core-fucosylated biantennary structure carrying a bisecting GlcNAc and therefore, only a limited amount of pure P0 could be released from the protein backbone, had to be limited as much as possible in order to prevent the loss of precious material. As

**DISCUSSION**

In this study, we describe the detailed structural characterization of the N-linked glycans present on bovine peripheral myelin P0 using state-of-the-art NMR and mass spectrometry techniques. These glycans occur in addition to the core-fucosylated biantennary structure carrying a bisecting GlcNAc and the HNK-1 epitope on the Man-4' branch that was characterized previously by us (17). The isolation of myelin P0 proved to be extremely laborious; and therefore, only a limited amount of pure P0 could be obtained. Fractionation of the carbohydrate pool, after being released from the protein backbone, had to be limited as much as possible in order to prevent the loss of precious material. As
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a result, most of the 10 fractions contained several structurally related glycoforms and had to be analyzed as mixtures. To perform the structural analysis successfully, ultrasensitive analytical methods capable of providing detailed structural information on such complex samples had to be employed. The newly developed nano probe MAS 1H NMR technique allows the structural analysis of nanomolar quantities of material in solution. So far, this technique has scarcely been applied in structural carbohydrate analysis (38-40). The nano tube, which holds a volume of 40 µl, is normally spun at ~2 kHz at the magic angle (54.7°). Spinning at the magic angle removes magnetic susceptibility-induced line broadening and/or contributions from homonuclear dipolar couplings. With the novel probes and the use of gradients, it is possible to apply existing solution state NMR techniques for solving challenging structural problems. In the case of the P0 glycoprotein, the MAS 1H NMR technique was complemented by sensitive mass spectrometric analyses.

The structures identified were for 75 ± 5% of the hybrid-type and for 25 ± 5% of the biantennary complex-type, carrying a distinct variety of structures at the Man-4 branch. All structures were core-fucosylated. Interestingly, Burger et al. (1), who studied P0 from human sciatic nerve by Western blot analysis and affinity chromatography, observed predominantly tri- and tetraantennary oligosaccharides (~80%), in addition to biantennary (10%) and oligomannose and/or hybrid (10%) oligosaccharides. This result may point to species-specific structures. In a study on human material, Field et al. (3) used enzymatic or chemical degradation in conjunction with chromatographic techniques for identification. They described the presence of HNK-1-positive carbohydrates, probably hybrid-type structures, containing either one sulfate residue (80%) or three sulfate residues (20%) in combination with sialylation.

Among the different structures we observed, the HNK-1 epitope (3-O-SO3H-GlcUA(β1–3)Gal(β1–4)GlcNAc), detected in fractions Q2.8–10, has been well documented. This major carbohydrate epitope is prominently present in the nervous system and is carried by a variety of cell-surface glycolipids as well as glycoproteins (41). The antigen has been found to be one of the factors responsible for the precise cell adhesion and recognition processes that underlie the interaction of neural cells (42). It has also been implicated in interactions with laminin and peripheral nerve regeneration (43, 72–77). This carbohydrate antigen, also found in the retina and on HNK cells, is the main target of IgM molecules from patients with peripheral demyelination neuropathies (44, 78). Especially the sulfate ester within the epitope seems to play an essential role in the myelin assembly during development (45) and outgrowth of neurites (46). The sulfation of glucuronic acid, which occurs at the onset of myelination and regeneration and ceases after development, is mediated by a highly specific HNK-1 sulfotransferase (47, 48). In this context, the presence of the 6-O-sulfoglcNAc oligosaccharides (fractions Q1.4–2.10) and specifically the 6-O-sulf-HNK-1 epitope, observed in fraction Q2.10 is interesting since it implies another sulfotransferase activity. Of the various 6-O-sulf-GlcNAc sulfotransferases identified thus far (49–52, 79), only one report describes the existence of a nervous system-involved sulfotransferase (53), which showed a high degree of homology to a family of 6-O-sulfotransferases, some of which are expressed also in brain (50, 54, 80, 81).

The observation of the 6-O-sulfosialyl-Lewis X and non-fucosylated precursors as capping epitopes was surprising. The terminal Neu5Ac(α2–3)Gal(β1–4)[6-O-SO3H]GlcNAc structure is very uncommon and has been found only in N-glycans of the glycoprotein family (ZP3) from zona pellucida (55). The 6-O-sulfosialyl-Lewis X epitope has been identified as a potent inhibitor of the leukocyte adhesion molecule L-selectin (18), and its structure, function, and biosynthesis have been described by Kimura et al. (56). It is intriguing that we found its presence on the very same molecule that carries the HNK-1 antigen that also binds to L-selectin (57, 58). Previously, it has been suggested that the sulfoglucuronylparagloboside (containing the HNK-1 epitope) acts as the ligand for L-selectin in inflammatory disorders of both the central and peripheral nervous systems to regulate the invasion of activated lymphocytes into the brain (58). It is suggested that the expression of the 6-O-sulfosialyl-Lewis X structure serves the same purpose.

The α-galactosyl epitope (59) in combination with a 6-O-sulfated GlcNAc (Galα1–3)Gal(β1–4)6-O-SO3H-GlcNAc, as observed in fraction QN.3, has not been described before in glycoproteins. The non-sulfated analog has been found on the N-linked glycans of mouse oocyte ZP3 (60), porcine thyroglobulin (31), and IgG1 antibodies produced by murine and transfected cell subclones (61) and has also been observed on gangliosides (33). The Gal(α1–3) linkage to the reducing terminal GalNAc in O-linked glycans was found to be present in significant quantities only in brain (62) and nervous tissue glycoproteins (63). Whether the presence of this α-galactosyl epitope, which has been identified as the major xenoantigen in pig-to-man transplants, is due only to the availability of the appropriate enzyme and donor substrate or whether it is functionally significant remains to be clarified. In fraction Q1.7 a second terminal structure was observed (Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1–4)[6-O-SO3H]GlcNAc), which is completely new in N-linked glycans from glycoproteins. The distal terminal epitope has been observed on glycosphingolipids from bovine brain (35) and frog brain (64) and in a variety of glycoproteins (65, 82–88), although never in combination with a sulfated GlcNAc. This structure, when present in higher polymeric forms as the so-called unusual polysialic acid associated with the neural cell adhesion molecule (N-CAM), is known to function in cell adhesion, differentiation, signal transduction, and surface expression of stage-specific developmental antigens. It indicates the expression of a sialyltransferase capable of generating the α2,8 linkage (66). Recently, Sato et al. (67) demonstrated the presence of this non-sulfated structure on many mammalian brain glycoproteins, using specific antibodies. It is more prominent in adult than in embryonic porcine brain tissue, suggesting a developmental regulation.

In summary, the nano probe MAS 1H NMR spectroscopic and mass spectrometric techniques employed in this study, being at least a factor of 5 more sensitive than traditional methods, have demonstrated to be very powerful tools for the characterization of the N-glycans on the P0 glycoprotein from bovine peripheral myelin. The identification of the epitope library may bring us one step closer to the understanding of the relationship between the carbohydrates present and their putative functions in neural tissue (68–71). The observation of predominantly hybrid- and biantennary complex-type structures is remarkable. It is, however, very likely that the already mentioned spatiotemporally regulated and species-dependent expression causes the variety of epitopes observed. Although the glycosylation machinery is apparently fully operational, no higher antennary structures have been observed in bovine material, which might point toward a very subtle modulation of the glycan heterogeneity by the down-regulation of specific glycosyltransferases. The functional significance of the observed structural diversity poses a new challenging question.

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