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GLYCOPEPTIDE FRACTIONS PREPARED FROM PURIFIED CENTRAL AND PERIPHERAL RAT MYELIN

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Summary

Myelin was purified from rat brain and sciatic nerve after in vivo labeling with [^{3}H]fucose and [^{14}C]glucosamine to provide a radioactive marker for glycoproteins. The glycoproteins in the isolated myelin were digested exhaustively with pronase, and glycopeptides were isolated from the digest by gel filtration on Bio-Gel P-10. The glycopeptides from brain myelin separated into large and small molecular weight fractions, whereas the glycopeptides of sciatic nerve myelin eluted as a single symmetrical peak. The large and small glycopeptide fractions from central myelin and the single glycopeptide fraction from peripheral myelin were analyzed for carbohydrate by colorimetric and gas liquid chromatographic techniques. The glycopeptides from brain myelin contained 2.4 μg of neutral sugar and 0.59 μg of sialic acid per mg total myelin protein, whereas sciatic nerve myelin glycopeptides contained 10 μg of neutral sugar and 3.8 μg of sialic acid per mg total protein. Similarly, the gas-liquid chromatographic analyses showed that the glycopeptides from peripheral myelin contained 4- to 7-fold more of each individual per mg total myelin protein than those from central myelin. Most of the sialic acid and galactose in the glycopeptides from central myelin were in the large molecular weight fraction, and the small molecular weight glycopeptides contained primarily mannose and *N*-acetylglucosamine. The considerably higher content of glycoprotein-carbohydrate in peripheral myelin supports the results of gel electrophoretic studies, which indicate that the major protein in peripheral myelin is glycosylated while the glycoproteins in purified central myelin are quantitatevely minor components.

Introduction

There is currently a great deal of interest in the structure and function of surface membrane glycoproteins [1]. However, other than the major glycopro-

tein of the erythrocyte [2], our knowledge of the chemistry of membrane glycoproteins is limited. Myelin, which is derived from the surface membrane of glial cells, is generally considered to have a relatively simple biochemical composition and has played a central role as the current concepts of membrane structure have developed. Highly purified myelin preparations from rat brain and sciatic nerve contain glycoproteins [3—6]. These glycoproteins were first detected on polyacrylamide gels by periodic acid-Schiff staining or by radioactive counting following in vivo labelling with specific sugar precursors. The glycoproteins in purified central myelin have high molecular weights and are quantitatively minor components [3]. The most prominent of these high molecular weight glycorproteins in purified central myelin is a true component of myelin or other oligodendroglial-derived membranes in the tissue [3,7—11]. Isolated sciatic nerve myelin does not contain this high molecular weight glycoprotein which is associated with central myelin. However, the major protein of peripheral myelin, with a molecular weight of approx. 30 000, is glycosylated [4—6]. In order to obtain qualitative and quantitative information about the sugars in these myelin-associated glycoproteins, we isolated glycopeptide fractions from rat brain and sciatic nerve myelin, and analyzed them for carbohydrate by colorimetric and gas-liquid chromatographic techniques. These results have been presented in preliminary form [12,13], and provide the first quantitative comparison of glycoprotein-carbohydrate in central and peripheral myelin.

Experimental procedures

Animals and materials. Male Osborne-Mendel rats (200—250 g) were supplied by the Veterinary Resources Branch at N.I.H. L-[1,5.6-^{3}H]fucose and D-[U-^{14}C]glucosamine were purchased from the New England Nuclear Corp. Unlabeled L-fucose and "pronase" (Type VI protease from *Streptomyces griseus*) were purchased from the Sigma Chemical Co. Acrylamide and *N,N'*-methylenebisacrylamide were purchased from Eastman Organic Chemicals, and the sodium dodecyl sulfate was Sequanal grade from Pierce Chemical Co. Bio-Gel P-10 (100—200 mesh) and Bio-Gel P-2 (100—200 mesh) were obtained from Bio-Rad Laboratories. Trimethylchlorosilane, hexamethyldisilazane, and silylation grade pyridine were obtained from Pierce. The stationary support for gas-liquid chromatography was methyl silicone obtained from Applied Science.

Injection of animals and purification of myelin. Rat brain glycoproteins were labeled by intracranial injection of 20 μCi of [^{3}H]fucose (125 Ci/mol) and 8 μCi [^{14}C]glucosamine (228 Ci/mol) as previously described [3]. Rat sciatic nerves were surgically exposed and injected with 10 μCi of [^{3}H]fucose and 4 μCi of [^{14}C]glucosamine per nerve as described in an earlier publication [4]. In some experiments, the animals were injected only with [^{3}H]fucose. All animals were killed 16—20 h after injection, and the brains and sciatic nerves were removed and rinsed in 0.32 M sucrose. Myelin was purified from the brains and nerves by the procedure of Norton and Poduslo [14]. The sciatic nerves were frozen with liquid N_2 and pulverised in a mortar and pestle [15] before preparing the homogenate from which the myelin was purified. The purified myelin preparations were suspended in water, sampled for protein determination, and

freeze-dried. The myelin was stored under vacuum and desiccated at −20°C until used.

Analytical gel electrophoresis. Portions of lyophilized myelin were partially delipidated with diethylether/ethanol (3 : 2, v/v) [16]. The proteins were solubilized in a solution containing 1 g sodium dodecyl sulfate, 1.5 g dithiothreitol, and 8 g sucrose per 100 ml [17], in a boiling water bath for 3 min. Electrophoresis was on 10% polyacrylamide gels as previously described [3] and the proteins were stained with Fast Green [16]. The gels were sliced into 2.2-mm sections with a razor blade and template, and the slices corresponding to the major stained bands were noted. Radioactivity in the slices was determined as in Method A of Quarles et al. [3].

Preparation and isolation of glycopeptides. Portions of lyophilized brain myelin containing between 18 and 33 mg of protein were extracted two times with chloroform/methanol/0.1 M KCl (40 : 20 : 3, v/v). The extraction was done by suspending the myelin in the solvent at a concentration of 2 mg protein per ml and stirring for 30 min. One-tenth volume of methanol was added and the insoluble residue was sedimented at 100 000 × *g* for 20 min. The residue was extracted a second time in the same way and suspended in 1.5 ml of H_2O with a small, glass hand-homogenizer. The suspension was adjusted to a final volume of 2.0 ml containing 0.1 M Tris · HCl buffer (pH 7.8) and 2.5 mM $CaCl_2$. The sample was incubated at 37°C for 5 days in the presence of saturating amounts of toluene to prevent bacterial growth. 50 µl of a 3 mg/ml pronase solution were added at the beginning of the incubation, and 25-µl additions were made on days 2 and 4. At the end of the incubation, the soluble glycopeptides were separated from the insoluble residue by low speed centrifugation. Much of the residue consisted of lipid and floated. The soluble fraction was removed from the residue with a Pasteur pipette. The residue was washed with 1 ml of water. The combined soluble fraction and wash was concentrated to about 1 ml by partial lyophilization, and applied to a column of Bio-Gel P-10 as described below.

Glycopeptides were prepared from sciatic nerve myelin in the same way except that less myelin was used, varying between 7 and 12 mg of protein. Only 20 µl of the 3 mg/ml pronase solution were added at the beginning of the incubation and subsequent additions were 10 µl.

The soluble glycopeptides were purified by gel filtration on a column of Bio-Gel P-10 (90 × 1.5 cm). The void volume was determined with Blue Dextran and the total volume with radioactive fucose before applying the sample. The column was eluted with 0.1 M NaCl and 2.0-ml fractions were collected. The positions of the labeled glycopeptides were determined by counting 200-µl aliquots of each fraction. The tubes containing the glycopeptides were combined as described in Results and lyophilized. The dried glycopeptide fractions were suspended in 1.2 ml of water and desalted by passage through a small column of Bio-Gel P-2 (25 × 1 cm). The labeled glycopeptides were located by counting 25-µl aliquots and the tubes with radioactivity were combined, and lyophilized. Each glycopeptide fraction was suspended in 1.0 ml of water and aliquots were taken for analysis as follows: one-fifth for total neutral sugar, one-fifth for sialic acid, one-half for gas-liquid chromatography, and 2% for liquid scintillation counting. All analyses were calculated as the amount of

sugar per mg of total myelin protein at the beginning of the experiment and were corrected for aliquots taken during the isolation of glycopeptides.

Colorimetric analyses. Total myelin protein was determined by the method of Lowry et al. [18] at the time myelin was purified. After color development, the samples were extracted with chloroform to eliminate turbidity due to lipids. Human serum albumin was used as a protein standard. Total neutral sugar was determined by the phenol-H_2SO_4 method of Dubois et al. [19], using galactose as a standard. Hexosamines were determined with *p*-dimethylaminobenzaldehyde [20] and sialic acid by the thiobarbituric acid procedure [21]. In preliminary experiments, these methods were applied to the whole chloroform/methanol-extracted protein residue of myelin. In these early experiments, the neutral sugar fraction was obtained by hydrolysis in 1 M H_2SO_4 for 6 h at 100°C and passage through Dowex-50 and Dowex-1 [22]. Hexosamines were released by 4 M HCl at 100°C for 8 h and eluted from Dowex-50 as described by Boas [23]. Sialic acid was released by 0.05 M H_2SO_4 at 80°C for 90 min. In the experiments with the purified glycopeptides, the phenol-H_2SO_4 procedure was done directly on the intact glycopeptides, whereas the sialic acid was hydrolyzed as described above.

Gas-liquid chromatography. Individual sugars in the isolated glycopeptides were determined as trimethylsilyl derivatives essentially as described by Clamp et al. [24]. The aliquot taken for gas-liquid chromatography analysis was lyophilized and the methylglycosides were released by treating it with 1 ml of 1 M methanolic · HCl at 85°C for 16 h in a sealed ampoule under N_2. After reacetylation, trimethylsilyl derivatization was done with 50 µl of trimethylchlorosilane/hexamethyldisilazane/pyridine (1 : 1 : 5, v/v). 5 µl of this solution were injected into a column of methylsilicone in a Perkin-Elmer 900 gas chromatograph. A temperature gradient of 2°C per min from 140 to 230°C was applied. The amounts of individual sugars were calculated from the areas under the peaks using mannitol, which was added to each sample before the methanolysis, as an internal standard.

Results

Isolation of glycopeptides from central and peripheral myelin preparations

Myelin was purified from the brains of rats which had been injected intracranially with [^{3}H]fucose and [^{14}C]glucosamine in order to label glycoproteins. Polyacrylamide gel electrophoresis of myelin proteins in the presence of sodium dodecyl sulfate showed a group of labeled high molecular weight glycoproteins in which the distributions of ^{3}H and ^{14}C were very similar (Fig. 1A). The most prominent radioactive glycoprotein did not correspond to any of the major myelin protein bands which were stained with fast green. The myelin was delipidated and the glycoproteins were converted to glycopeptides by exhaustive pronase digestion as described in Experimental Procedures. When the soluble glycopeptides were fractionated on Bio-Gel P-10, both the [^{3}H]fucose- and [^{14}C]glucosamine-labeled glycopeptides emerged from the column as two broad peaks in which the ^{3}H/^{14}C ratio varied (Fig. 2A). The tubes making up the two peaks were combined as shown in the figure to give the large and small molecular weight glycopeptide fractions which were desalted by passage through a col-

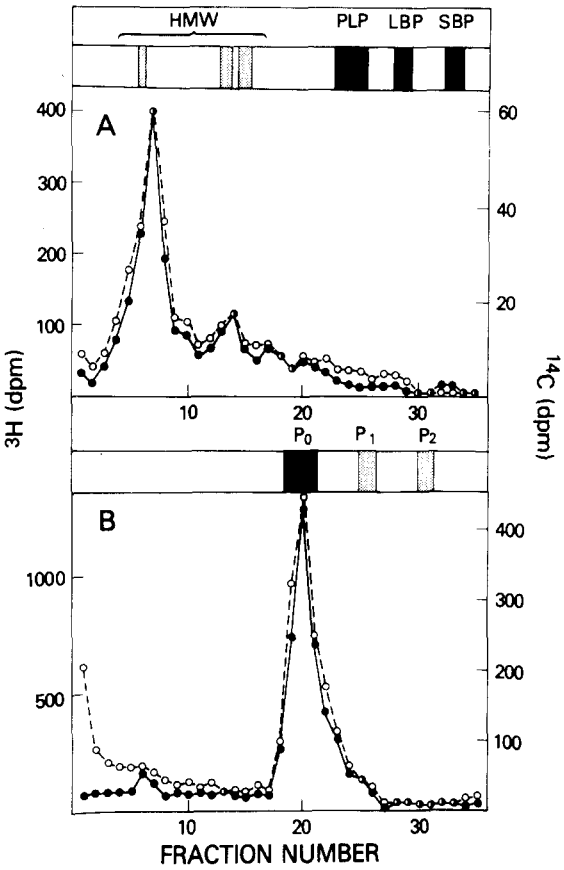

Fig. 1. Polyacrylamide gel electrophoresis of myelin proteins. Myelin was purified from rat brain and sciatic nerve after in vivo labeling of glycoproteins with [^{3}H]fucose and [^{14}C]glucosamine. The myelin proteins were solubilized with sodium dodecyl sulfate and electrophoresed in 10% polyacrylamide gels. The proteins were stained with Fast Green and the gels were cut into 2.2-mm segments for radioactive counting. The fractions corresponding to the most prominent stained bands were noted while slicing the gel, and the drawings at the top of each panel show the positions of the major proteins relative to the radioactivity. The lower part of each panel shows the distribution of ^{3}H (●———●) and ^{14}C (○- - - - - -○) on the gels. (A) Brain myelin: HMW, high molecular weight proteins; PLP, proteolipid protein; LBP, large basic protein; SBP, small basic protein. (B) Sciatic nerve myelin. The main bands are labeled according to Greenfield et al. [15]. P_0 is the major protein, and P_1 and P_2 are basic proteins.

umn of Bio-Gel P-2. These two glycopeptide fractions were isolated from four separate preparations of rat brain myelin and used for carbohydrate analyses.

Myelin was also purified from rat sciatic nerves which had been injected with [^{3}H]fucose and [^{14}C]glucosamine. Polyacrylamide gel electrophoresis showed that both isotopes were incorporated primarily into the major protein of peripheral myelin which stained intensely with fast green (Fig. 1B). In addition, both isotopes labeled a component which ran just in front of the major band on the gels, so that there was a shoulder of radioactivity on the leading edge of the major peak. Glycopeptides were prepared from peripheral myelin in the same manner as from central myelin. When applied to a column of Bio-Gel P-10, the glycopeptides emerged as a single symmetrical peak in which the ^{3}H

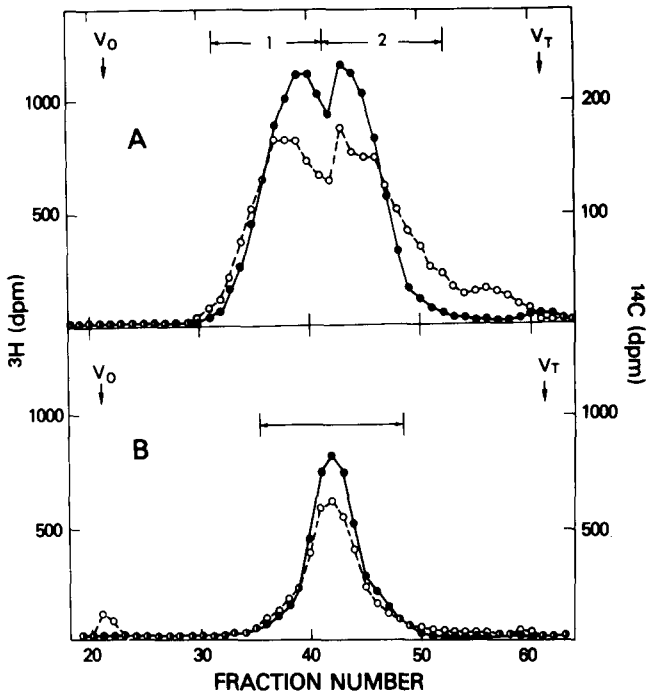

Fig. 2. Gel filtration of glycopeptides prepared from myelin. The [^{3}H]fucose and [^{14}C]glucosamine labeled glycoproteins shown in Fig. 1 were converted to glycopeptides by pronase digestion, and the glycopeptides were fractionated on Bio-Gel P-10. V_0, void volume; V_T, total volume; •———•, ^{3}H; ○------○, ^{14}C. (A) Glycopeptides from brain myelin: The designated fractions were pooled to give the large [1] and small [2] glycopeptide fractions. (B) Glycopeptides from sciatic nerve myelin: the designated fractions were pooled to give the glycopeptide fraction of peripheral myelin.

and ^{14}C had very similar profiles (Fig. 2B). The tubes making up the radioactive peak were combined as shown in the figure to give the glycopeptide fraction of peripheral myelin. Carbohydrate analyses were done on glycopeptide fractions obtained in this manner from four separate preparations of sciatic nerve myelin.

The distribution of radioactive fucose was followed during the preparation of glycopeptides from peripheral and central myelin to give an indication of the recovery of glycopeptide-carbohydrate in the glycopeptide fractions. The percentage of radioactive fucose in the lipid extracts was only 7.4 ± 0.6% for central myelin and 4.7 ± 0.4% for peripheral myelin. After pronase digestion, 3.8 ± 1.3% of the radioactivity in central myelin and 6.3 ± 1.3% of the radioactivity in peripheral myelin remained with the insoluble residue. The recoveries of radioactive fucose in the final glycopeptide fractions used for carbohydrate analysis, after purification on Bio-Gel P-10 and desalting on Bio-Gel P-2, were 81 ± 5.3 and 78 ± 3.2% for central and peripheral myelin, respectively. These recoveries are expressed as percentage of [^{3}H]fucose in the whole soluble glycopeptide fraction immediately after pronase digestion. The corresponding recoveries of [^{14}C]glucosamine were 74% for central myelin and 89% for peripheral myelin.

TABLE I

COLORIMETRIC ANALYSES OF SUGARS IN GLYCOPEPTIDES PREPARED FROM DELIPIDATED MYELIN

The values are given in μg of glycopeptide-carbohydrate per mg of total myelin protein and are the means ± S.E. for four separate preparations of rat brain and sciatic nerve myelin.

	Rat brain			Rat sciatic nerve
	Large glycopeptides	Small glycopeptides	Total	Total
Neutral sugar	1.1 ± 0.14	1.3 ± 0.08	2.4 ± 0.22	10 ± 0.8
Sialic acid	0.50 ± 0.07	0.09 ± 0.019	0.59 ± 0.067	3.8 ± 0.72

Colorimetric sugar analyses

The amounts of neutral sugar in the glycopeptide fractions purified from central and peripheral myelin are shown in Table I. The amount of glycopeptide-sugar per mg of total myelin protein was bout 4-fold higher in peripheral myelin than in central myelin. In central myelin, there was slightly more neutral sugar in the small glycopeptide fraction than in the large fraction. Similarly, the total amount of sialic acid as determined by the thiobarbituric acid procedure was about 6-fold higher in the glycopeptides isolated from peripheral myelin than in those from central myelin (Table I). For central myelin, over 80% of the sialic acid was in the larger glycopeptide fraction.

Gas-liquid chromatographic analyses of individual sugars

Table II shows the amounts of individual sugars in the glycopeptide fractions prepared from rat brain and rat sciatic nerve myelin, respectively. The data support the colorimetric analyses in showing that peripheral myelin yielded considerably larger amounts of glycopeptides than central myelin. It can be seen that the glycopeptides of peripheral myelin contained 4—7-fold more of each individual sugar than the glycopeptides of central myelin. The large molecular weight glycopeptide fraction of central myelin contained most of the galactose

TABLE II

GAS-LIQUID CHROMATOGRAPHIC ANALYSES OF GLYCOPEPTIDE FRACTIONS PREPARED FROM RAT BRAIN AND SCIATIC NERVE MYELIN

Results are given as nmol of each sugar per mg of total myelin protein. The values are means ± S.E. for three separate preparations of brain myelin and four separate preparations of sciatic nerve myelin.

	Brain			Sciatic nerve
	Large glycopeptides	Small glycopeptides	Total	
Fucose	0.61 ± 0.07	0.73 ± 0.20	1.3	8.7 ± 1.4
Mannose	1.8 ± 0.28	3.6 ± 0.50	5.4	37 ± 3.3
Galactose	1.9 ± 0.26	0.76 ± 0.11	2.7	14 ± 0.9
N-Acetylglucosamine	2.9 ± 0.26	2.9 ± 0.62	5.8	28 ± 4.6
N-Acetylneuraminic acid	1.4 ± 0.13	0.24 ± 0.01	1.7	11 ± 1.2

and sialic acid, while the small glycopeptide fraction contained primarily mannose and *N*-acetylglucosamine.

Little or no *N*-acetylgalactosamine could be detected in any of the glycopeptide fractions from central or peripheral myelin and could not have been present in amounts greater than 5% of the level of *N*-acetylglucosamine. Small and variable amounts of glucose were detected in all glycopeptide fractions. However, in control experiments in which pronase incubations without myelin were carried through the gel filtration steps, similar levels of glucose were detected, suggesting that the glucose is not a component of the myelin glycopeptides. Some of the glucose may derive from the pronase preparation [25,26]. None of the other sugars were detected in these control experiments.

Discussion

The purpose of this investigation was to determine the identity and quantity of the sugars in glycoproteins of myelin purified from rat brain and rat sciatic nerve. We used in vivo labeling with [^{3}H]fucose and [^{14}C]glucosamine to provide a sensitive marker for the glycoproteins in purified myelin and for the glycopeptides prepared from them. Electrophoresis of the labeled glycoproteins on sodium dodecyl sulfate gels confirmed earlier studies [3,4], showing that the most prominent labeled component in brain myelin does not correspond to one of the major proteins, whereas, the sugar precursors were incorporated into the major stained protein of sciatic nerve myelin.

Some technical problems had to be solved in order to obtain reliable values for glycoprotein-carbohydrate in myelin. It was necessary to remove the large amount of glycolipid in myelin from the sample for analysis, while retaining essentially all of the carbohydrate in glycoproteins. Simple chloroform/methanol extraction was not satisfactory for two reasons. First, although most of the myelin glycoproteins are insoluble in chloroform/methanol, we found, that in lyophilized central myelin, the proportion of fucose-labeled glycoproteins which was extracted into chloroform/methanol (2 : 1, v/v) varied between 10 and 35% from preparation to preparation. The chloroform/methanol-soluble radioactivity showed a pattern of high molecular weight glycoproteins on sodium dodecyl sulfate gels which was very similar to the glycoproteins in the insoluble residue, indicating that the glycoproteins were partially soluble in the organic solvents. This problem was overcome by including 5% by volume of 0.1 M KCl in the chloroform/methanol used for lipid extraction, since this kept the amount of lipid-soluble glycoprotein below 10% of the total. The second technical difficulty was that a small proportion of the large amount of glycolipid in myelin remained with the protein residue even after extensive chloroform/methanol extraction. As a result, neutral sugar determinations on the lipid extracted protein residue were artifactually high. Thus, we found 7.3 ± 1.57 (n = 3) μg of neutral sugar per mg total protein for brain myelin which is 3-fold higher than the values obtained for the isolated glycopeptides (Table I). This illustrates the inadequacy of the whole delipidated myelin fraction as a sample for glycoprotein analysis. In order to obtain reliable carbohydrate data, we found it necessary to convert the glycoproteins to glycopeptides and purify the glycopeptides by gel filtration. After pronase digestion, most of the remaining

lipids were removed with the insoluble residue, and a small portion which remained in suspension were excluded from the Bio-Gel P-10 column. In contrast to the neutral sugars, the values obtained for amino sugars and sialic acid on the chloroform/methanol-insoluble residue of central myelin were in reasonable agreement with those obtained for the purified glycopeptides.

The levels of glycoprotein-carbohydrate which we found in purified rat brain myelin per mg of total myelin protein are only 15—35% of the levels reported for whole rat brain [27—29]. Thus, the amount of glycoprotein in purified myelin is low in comparison to other membranes in brain. This is consistent with earlier staining and labeling experiments which indicated that the glycoproteins in central nervous system myelin are quantitatively minor components [3]. It must be emphasized that the carbohydrate composition reported here is for glycopeptides prepared from the whole mixture of high molecular glycoproteins in purified myelin and only the most prominent of these has definitively been shown to be associated with myelin or other oligodendroglial-derived membranes in the tissue [7—10]. It is likely that some of the other glycoproteins in purified myelin are similarly associated with myelin in the tissue [3], but this has not been established. Since the glycopeptides were prepared from a mixture of glycoproteins, it is not surprising that the $^{3}H/^{14}C$ ratio varied considerably as the glycopeptides were eluted from the column of Bio-Gel P-10 indicating a substantial degree of heterogeneity in both the large and small molecular weight fractions. The enrichment of sialic acid and galactose in the high molecular weigh glycopeptides, and the preponderance of mannose and *N*-acetylglucosamine in the small molecular weight fraction is similar to results reported for glycoproteins of whole brain and other membranes in brain [28—31]. Although, there is only a small amount of the most prominent glycoprotein in the whole myelin fraction, we have shown that it is selectively concentrated in heavy myelin subfractions which are probably enriched in membranes which are transitional between compact myelin and the oligodendroglial plasma membrane [32,33]. Also, a surface labeling procedure with galactose oxidase and tritiated $NaBH_4$ showed that the glycoprotein is at least partially localized on the surface of the myelin-oligodendroglial complex [11]. Therefore, the concentration of the glycoprotein in these surface, oligodendroglial-derived, membranes may be substantially higher and comparable with the concentrations of glycoproteins in other surface membranes. However, as the glial plasma membrane is spiraled and compacted to form myelin, the glycoprotein would be diluted by the addition of the major structural proteins of myelin and is present in only low concentration in the whole myelin fraction.

Several years ago, we showed that the major protein of rat sciatic nerve myelin is a glycoprotein [4]. This conclusion was based on fucose labeling and periodic acid-Schiff staining of peripheral myelin proteins which were separated by polyacrylamide gel electrophoresis and on the qualitative demonstration of sugars which are characteristic of glycoproteins. Similar observations have been made by a number of other laboratories [5,6,34—36]. Although there is currently some uncertainty in the literature concerning the distribution of carbohydrate between the major stained band on sodium dodecyl sulfate gels and some minor components in the same region of the gel [6,36], all studies indicate that the major protein (P_0 in the terminology of Greenfield et al. [15]), is

glycosylated [4–6,34–37]. Therefore, our finding of a substantially higher content of glycoprotein-carbohydrate in sciatic nerve myelin than in brain myelin is not unexpected, and confirms the concept that the glycoproteins of peripheral myelin are quantitatively more important components. The fact that the glycopeptides from peripheral myelin emerged from the Bio-Gel P-10 column as a single symmetrical peak in which the [^{3}H]fucose and [^{14}C]glucosamine had similar profiles suggests a considerably greater degree of homogeneity than in the central nervous system myelin glycopeptides. The peripheral myelin glycopeptides are sulfated [40] as are the large glycopeptides from brain myelin [41]. Histochemical studies using concanavalin A binding [38] and correlative biochemical and electron microscopic studies during isoniazid-induced nerve degradation [39] have indicated that the major glycoprotein is localized in the less dense, intraperiod line of peripheral myelin. It has been suggested that the high content of glycoprotein-carbohydrate at this site could account for the greater separation of the two membranes at the intraperiod line in peripheral myelin in comparison to central myelin [38].

The data reported here show that both central and peripheral myelin contain many of the sugars which are characteristic of glycoproteins and provide the first quantitative comparison between the amounts of the various sugars in central and peripheral myelin. The results confirm the indications from gel electrophoretic studies that the glycoproteins of peripheral myelin are present in larger amounts than those in central myelin.

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