Sulfated Glycoproteins, Glycolipids, and Glycosaminoglycans from Synaptic Plasma and Myelin Membranes: Isolation and Characterization of Sulfated Glycopeptides†

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ABSTRACT: In this report we provide biochemical evidence that a highly purified synaptic plasma membrane fraction derived from rat brain, after intraventricular injection of ^{35}S-labeled sodium sulfate, is enriched in a number of large sulfated glycoproteins compared with a purified myelin fraction studied concurrently. A fraction of the detergent-solubilized sulfated glycoprotein bound specifically to concanavalin A–Sepharose. In addition, we have identified the ^{35}S-labeled lipid-soluble material in these membrane fractions as cerebroside sulfate. The sulfated protein in the lipid-extracted membranes was shown to consist predominantly of a class of glycoproteins containing sulfate in ester linkage to oligosaccharide chains, which are differentiated structurally from the sulfated glycosaminoglycans of brain. These two classes of sulfated macromolecules were distinguished from one another by several chemical and physical parameters. We present the chemical characterization of the sulfated glycopeptides derived from synaptic plasma and myelin membranes by extensive proteolytic digestion after quantitative removal of cerebroside sulfate. Membrane-associated glycosaminoglycans, either specifically or adventitously associated with these neuronal membranes, were quantitatively precipitated and identified.

The existence of sulfated glycoproteins, that is, glyco conjugates containing sulfate that are structurally distinct from sulfated glycosaminoglycans of the intercellular matrix, was demonstrated in sheep colonic mucin (Kent and Marsden, 1963). Investigators (Margolis, 1967; Margolis and Margolis, 1970; Katzman, 1972; Brunngraber et al., 1973) have shown that sulfate exists in ester linkage to oligosaccharide chains in preparations of glycopeptides derived from whole rat brain. Sulfated glycopeptides were also prepared from whole bovine brain (Arima et al., 1969). Margolis and Margolis (1970) have isolated and characterized a sulfated glycopeptide fraction from whole rat brain following extraction of brain with chloroform–methanol. More recently, the predominant glycoprotein from rat brain myelin has been shown to be sulfated (Matthieu et al., 1975a). These investigators have also shown that a similar, if not identical, glycoprotein of rat sciatic nerve is sulfated (Matthieu et al., 1975b).

In general, sulfated glycoproteins have been identified and characterized only as the products of proteolytic digestion, glycopeptides, and appear to be either mucin-like components of the epithelium, or integral membrane components such as those we have identified as constituents of the synaptic plasma membrane. Sulfated glycoproteins with blood group H activity (Roussel et al., 1975) have recently been characterized after purification from the bronchial secretions of patients suffering from cystic fibrosis. Another unusual sulfated glycoprotein from chick chorioallantoic fluid (Choi and Meyer, 1974) contains fatty acids covalently linked to carbohydrate. The essential biological function of these mucin-like or integral membrane sulfated glycoproteins, not unlike glycoproteins in general, remains essentially unresolved.

Our interest lies in the nature and distribution of the sulfated glycoproteins in membranes, particularly those of the synaptic plasma membrane and their possible role in cell-surface regulation of metabolic function. Furthermore, sulfate has been recently shown to interact specifically with a number of pharmacological agents containing weak basic amines (Loh et al., 1974). It was of interest, therefore, to investigate the synaptic plasma membrane, the putative site of action of many neuropharmacological agents for the presence of sulfate-carbohydrate linkages in glycoprotein.

In this paper, we report the presence of high-molecular-weight sulfated glycoproteins, which are structurally distinct from brain glycosaminoglycans, in a purified synaptic plasma and myelin membrane fraction from rat brain. In addition, we present the purification and chemical characterization of the sulfated glycopeptide fractions derived from these membranes and have identified the sulfated glycolipid and glycosaminoglycans associated with these neuronal membrane fractions.

Experimental Procedures

Purified Glycosaminoglycans. These were generous gifts of the following: keratan sulfate, Dr. Karl Meyer, Yeshiva University; heparitin sulfate (heparan sulfate), Dr. Alfred Linker, University of Utah; hyaluronic acid, chondroitin sulfate, heparin and dermatan sulfate, Dr. Eugene Davidson, Pennsylvania State University.

Administration of Isotope. The solution of sodium [^{35}S]-sulfate (20 mCi, New England Nuclear) was evaporated and the residue dissolved in 0.66 ml of Krebs–Ringer buffer (pH 7.4) and administered by intraventricular injection of 10 μl to each of 45 eight-week-old, male, Sprague Dawley rats. The animals were sacrificed 24 h later and subcellular fractions prepared from labeled forebrains.

Preparation of Subcellular Membrane Fractions. Synaptosomes were prepared from labeled forebrains by the method of Cotman (1974), which incorporates additional washes of

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the crude mitochondrial pellet (P_2) to reduce microsomal contamination (Morgan et al., 1971) and a Ficoll–sucrose gradient for more efficient purification of synaptosomes. Gurd et al. (1974) have confirmed the significance of these two steps. Synaptic plasma membranes were isolated from synaptosomes obtained from Ficoll–sucrose gradients as described by Cotman (1974), with an additional modification of Cotman et al. (1974), in which the iodonitroneotetrazolium (INT[1])-treated synaptosomes were washed twice in 0.16 M sucrose and centrifuged at 30 000*g* for 15 min prior to being layered on sucrose gradients. In this modification, unbound formazan dye, enzymatically formed from the interaction of INT with mitochondrial succinic dehydrogenase, was more efficiently removed. The synaptic plasma membranes sedimenting at the 1.0 and 1.1 M sucrose interface were diluted with 3 volumes of distilled water and centrifuged at 100 000*g* for 25 min. Employing enzymatic assays for succinic dehydrogenase, (Na^+, K^+)ATPase, NADPH–cytochrome *c* reductase, cyclic 2′:3′-nucleotide 3′-phosphohydrolase, and monoamine oxidase, our laboratory has established that the synaptic plasma membranes obtained with this procedure are approximately 80% pure.

Myelin was obtained from Ficoll–sucrose gradients and purified with a series of hypotonic shocks and sucrose step gradients as reported by Norton and Podulso (1973). Norton, after morphological and enzymatic analysis, reported better than 95% purity of their isolated myelin fraction, and electron micrographs of our myelin fraction, prepared by the method of Cotman and Flansburg (1970), indicated a similar degree of purity. Microsomes were obtained from the supernatant (S_2) of the preparation used to isolate synaptosomes. The supernatant was centrifuged at 12 000*g* for 30 min and subsequently sedimented by centrifuging at 100 000*g* for 2 h. Mitochondria were collected from Ficoll–sucrose gradients and purified by resuspending in 0.32 M sucrose and layering on a 1.3 M sucrose gradient. A mitochondrial pellet was obtained after centrifuging at 53 000*g* for 90 min.

Synaptic Plasma Membrane Solubilization and Affinity Chromatography. Synaptic plasma membranes labeled with $Na_2{}^{35}SO_4$, or with $Na_2{}^{35}SO_4$ and L-[^{3}H]fucose, were extracted in 0.5% Triton X-100–0.01 M Tris-HCl (pH 8.0) at 2.25 mg of membrane protein/ml for 120 min at 4 °C with gentle homogenization. The soluble protein, which was recovered by centrifugation at 4 °C for 30 min at 100 000*g*, was subsequently dialyzed overnight against 0.05% Triton X-100–0.01 M NaCl–0.01 M Tris-HCl (pH 7.0). A 0.9 × 5 cm column of concanavalin A–Sepharose 4B (Pharmacia) was washed with water and then equilibrated with 0.05% Triton X-100–1 M NaCl–0.01 M Tris-HCl (pH 7.0) (Triton–Tris). The solubilized synaptic plasma membrane was applied to the conconavalin A column and washed with Triton–Tris, and washing was continued with 10 bed volumes of buffer or until the radioactivity and A_{280} returned to background. The elution buffer contained Triton–Tris in addition to 0.25 M methyl α-D-mannoside. The column was then washed with 1 M borate buffer (pH 7.4), to eliminate any residual specific or nonspecific binding.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in a discontinuous sodium dodecyl sulfate–polyacrylamide system (Maizel, 1971) with a 3% acrylamide stacking gel and a 7.5 or 15% separating gel. Stacking and separating gels were 1 and 10 or 1 and 12 cm, respectively, and the diameter of gels was 8 mm. Electrophoresis was performed in a modified Canalco Disk Electrophoresis Apparatus Model 1200 at a constant current of 3 mA/gel until cytochrome *c*, a reference protein, migrated to the end of the gel. Following electrophoresis gels were stained for protein (Fairbanks et al., 1971). The localization of glycoproteins on gels was accomplished by a modified Schiff–periodate method described previously (Simpson et al., 1974).

Extraction of Radioactivity from Gels. Gels were carefully sliced into 71 sections (1.4 mm) and slices were placed in scintillation vials containing 1 ml of 1% (w/v) sodium dodecyl sulfate. Vials were covered and shaken 24 h in a reciprocal shaker, and then heated to 60 °C for 2 h followed by incubation for 48 h with occasional shaking. After 12 ml of Scintiverse (Fisher) was added, the vials were shaken for an additional 24 h, allowed to incubate in the dark, and counted.

Liquid Scintillation Counting. Liquid scintillation counting was performed on a Beckman LS-150 liquid scintillation system at a window setting of 50 (lower) to 600 (upper) for [^{35}S]sulfate. Aqueous samples were mixed with 12 ml of Scintiverse. All membrane fractions were solubilized in 1 ml of 1% sodium dodecyl sulfate at 90 °C for 5 min prior to countings. Efficiency was determined with [^{35}S]sulfate of known specific activity and counts per minute (cpm) were adjusted to disintegrations per minute (dpm) accordingly.

Lipid Extraction of Membranes. Synaptic plasma or myelin membrane (24 mg of protein) was added to 150 ml of chloroform–methanol (2:1 v/v). The suspension was slowly homogenized in a Potter-Elvejhem tight-fitting homogenizer, five complete cycles. The suspension was centrifuged at 4 °C and 12 000 rpm for 30 min. The extraction was repeated and the supernatants were saved. The pellet was reextracted in a similar fashion a third time with 150 ml of chloroform–methanol (1:2 v/v).

Thin-Layer Chromatography. Lipid extracts were resolved on precoated silica gel 60 plates. Plates were developed at room temperature in a solvent of chloroform–methanol–water (35:15:2 v/v) and visualized by iodine vapor. Spots were scraped, extracted, evaporated to dryness, and counted. Cerebroside sulfate, containing either a hydroxylated or nonhydroxylated fatty acid, was well resolved from other lipid components in this system.

Sephadex LH-20 Chromatography. Lipid extracts were resolved on a 2 × 20 cm column of Sephadex LH-20 which had been equilibrated with chloroform. The column was run at room temperature and was standardized with respect to sample volume, flow rate, and fraction volume, for all experiments. Samples were resolved with solvent mixtures of increasing polarity according to the following schedule: chloroform, 40 ml; chloroform–methanol [(15:1 v/v) 10 ml], [(10:1 v/v) 10 ml], [(6:1 v/v) 10 ml], [(4:1 v/v) 40 ml], and [(1:1 v/v) 50 ml].

Cerebroside Sulfate Analysis. Cerebroside sulfate was measured employing the dye, Azure A (Kean, 1968). Cerebroside sulfate (Analabs) was employed as a standard. Specific activity is defined as dpm of cerebroside sulfate divided by the concentration of lipid in micrograms.

Cellulose Acetate Electrophoresis of Glycosaminoglycans. Glycosaminoglycans were resolved by dissolving the cetylpyridinium chloride precipitates in 0.5 M NaCl. Detergent in extracts was removed by dialysis against 0.5 M NaCl at 45 °C, followed by exhaustive dialysis against distilled water at 4 °C. Samples were concentrated and dissolved in an appropriate buffer.

[1] Abbreviations used: INT, iodonitroneotetrazolium; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)-aminomethane.

Electrophoresis on cellulose acetate strips was carried out in pyridine–acetic acid buffer (pH 3.5), at 10–15 mA/strip for varying periods of time in a Beckman Microzone Cell, Model R-101. Authentic standards or samples were detected with Alcian Blue. Profiles were obtained by scanning strips with a Beckman Microzone Densitometer Model R-110.

Preparation of Glycopeptides. Aliquots of lipid-extracted membrane, equivalent to 24 mg of synaptic plasma or myelin membrane protein, were homogenized in boric acid–borax buffer, 0.2 M (pH 7.4), containing 0.005 M $CaCl_2$ with tight-fitting ground-glass homogenizer. The volume was adjusted to 10 ml with incubation buffer and samnles suere treated for 10 min in an Aerograph ultrasonic bath (Branson), to assure homogenization. Protease type VI from *Streptomyces griseus* (Sigma) was added to a concentration of 0.5 mg/ml or 0.22 mg of enzyme/mg of membrane protein. Toluene was added and the tubes were sealed and incubated at 45 °C with occasional stirring and sonication for 48 h. Additional enzyme was added, 0.25 mg/ml, and the incubation continued for another 24 h. A third addition, 0.25 mg/ml, was made, and incubation continued for a total of 96 h. After cooling and centrifugation, undigested membrane protein and enzyme were precipitated by addition of trichloroacetic acid to a final concentration of 10%. After 6 h at 0 °C, the Pronase-soluble fraction was collected by centrifugation at 30 000 rpm for 30 min and then carefully adjusted to pH 7.0 with NaOH. The supernatant containing glycopeptides was exhaustively dialyzed at 4 °C against distilled water and then adjusted to 0.04 M with NaCl, an appropriate electrolyte concentration for precipitation of glycosaminoglycans. Carrier glycosaminoglycans were added to an aliquot of the supernatant at 37 °C and then titrated to a precipitation endpoint with cetylpyridinium chloride. A calculated amount of detergent was added to the residual supernatant to reach an identical endpoint. A 10% excess of cetylpryidinium chloride was added to assure complete precipitation, and the material was then incubated at 37 °C for 24 h. No turbidity was observed on incubation and the detergent-soluble fraction was recovered by centrifugation at 30 000 rpm for 30 min at room temperature. Excess detergent was precipitated from the supernatant with excess 2 M KSCN at room temperature, resulting in an insoluble detergent–thiocyanate complex. The soluble fraction containing glycopeptides was recovered by vacuum filtration with FG/A glass filters (Whatman) and excess KSCN was removed by exhaustive dialysis against glass-distilled water at 4 °C.

Glycopeptide Determination. Glycopeptide concentrations were determined using fluorescamine (Udenfriend et al., 1972). Bacitracin (Schwarz/Mann) served as a standard and relative fluorescence was recorded on an Aminco Bowman spectrofluorometer at an excitation wavelength of 390 nm and an emission wavelength of 485 nm.

Gel-Filtration Chromatography. A column, 1.2 × 60 cm, of Sephadex G-50 (fine) was equilibrated with 0.1 M pyridine acetic acid buffer (pH 5). The column was run at 4 °C and standardized with respect to sample volume, flow rate, and fraction volume. The void volume (V_0) was determined with Dextran Blue.

Ion-Exchange Chromatography. Glycopeptides were fractionated on Dowex 50 W-X4, 200–400 mesh, pyridine form. A column was equilibrated with 0.001 M pyridine formate (pH 3.0). Samples were dialyzed against 0.001 M pyridine formate buffer, applied to the column, and followed by elution with several column volumes of buffer. The column was then eluted with several column volumes of 0.01 M pyridine formate buffer which was followed by elution with buffer of increased ionic strength, 0.1 M at pH 3.

Dansylation and Polyamide Chromatography of Glycopeptide Hydrolysates. Glycopeptides were hydrolyzed in 6 N HCl (in vacuo) for 24 h at 100 °C. Hydrolysates were dried (in vacuo) over KOH and the dansyl chloride derivatives of amino acids and amino sugars were prepared according to Gray (1967). Dansyl derivatives were separated by two-dimensional chromatography (Woods and Wong, 1967). Individual derivatives were identified by concurrently chromatographing dansyl standards on the reverse side of plates.

Sulfate Hydrolysis and Resolution by Paper Electrophoresis. Sa ples of lyophilized glycopeptide were dissolved in 0.25 N HCl and hydrolyzed at 100 °C. Samples were taken at varying periods of time, 0–4 h, and spotted on paper for electrophoretic separation. Electrophoresis was performed on 5 × 35 cm paper strips (Whatman 3MM) in 0.05 M pyridine–acetic acid buffer (pH 3.8) at 10 mA/strip for 2–3 h in a Beckman paper electrophoresis cell (Durrum Type) Model R, Series D. Unlabeled glycopeptide was detected with ninhydrin or fluorescamine, while inorganic sulfate, or sulfated glycopeptide, was detected by counting 0.5-cm sections of strips.

Barium Sulfate Precipitation. Samples of labeled membrane or glycopeptide were hydrolyzed in 6 N HCl (in vacuo) at 100 °C for 24 h. The sulfate was precipitated as $BaSO_4$ with an excess of 0.1 M $BaCl_2$ (Kolthoff and Sandell, 1952) after addition of cold 250 μM carrier Na_2SO_4. Both supernatant and precipitate were collected by centrifugation and assayed for radioactivity.

Gas–Liquid Chromatography of Carbohydrates. The carbohydrate analysis was performed as described by Etchison and Holland (1974), which is a modification of the procedure of Clamp et al. (1971). Relative molar ratios of monosaccharides were determined by gas–liquid chromatography of the trimethylsilyl methyl glycosides. Peak areas were determined by cutting out tracings of peaks and weighing them on an analytical balance. Molar ratios were calculated relative to mannose employing molar relative response factors obtained by chromatography of authentic standards.

Uronic Acid and Neutral Carbohydrate Analysis. Analysis of neutral sugars was performed employing the phenol–sulfuric acid procedure (Dubois et al., 1956). Uronic acids were determined colorimetrically by the orcinol reactions (Brown, 1946) and by the modified uronic acid–carbazole reaction (Bitter and Muir, 1962).

Protein Determination. Protein was determined by the method of Lowry, with crystalline bovine serum albumin as a standard (Lowry et al., 1951). Membrane protein was determined by the alternate Lowry procedure which is described for microsomes (Albro, 1975).

Results

Characterization of Purified Membrane Fractions. To assess the purity of the membrane fractions and, in particular, to assure that the synaptic plasma membrane fraction was not contaminated with myelin that was reported to contain a sulfated glycoprotein, the purified membranes were subjected to polyacylamide gel electrophoresis in the presence of sodium dodecyl sulfate. For this purpose, 15% acrylamide gels were employed which permit resolution of the low-molecular-weight proteins which are distinctly characteristic of myelin. Although twice the amount of protein was applied to the gel containing synaptic plasma membrane compared with the myelin gel, it is apparent from the gel patterns seen in Figure 1, A and B, that the synaptic plasma membranes are essentially free of the

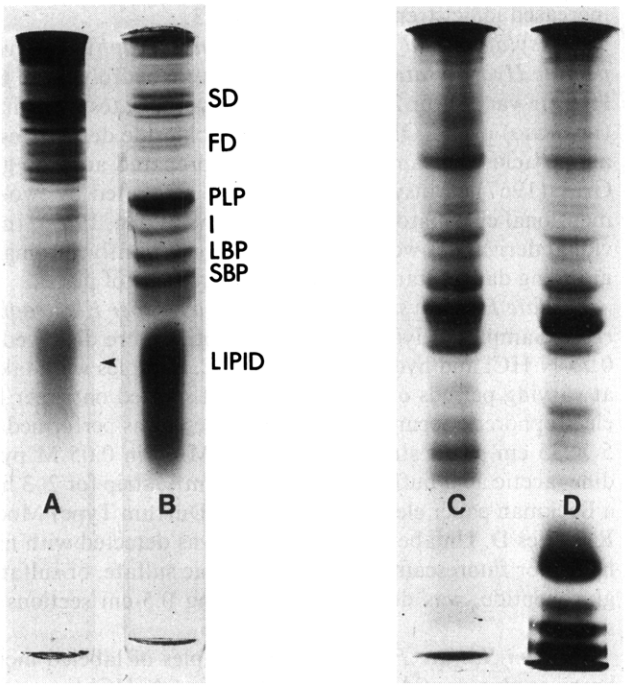

FIGURE 1: Purified (A) synaptic plasma membrane and (B) myelin, electrophoresed on 15% acrylamide gels in the presence of sodium dodecyl sulfate. Characteristic myelin proteins are: SD, slow doublet; FD, fast doublet; PLP, proteolipid protein; I, intermediate protein; LBP, large basic protein; SBP, small basic protein. Purified synaptic plasma membrane (C) and myelin (D), electrophoresed in the presence of sodium dodecyl sulfate on 7.5% acrylamide gels to resolve the high-molecular-weight membrane proteins.

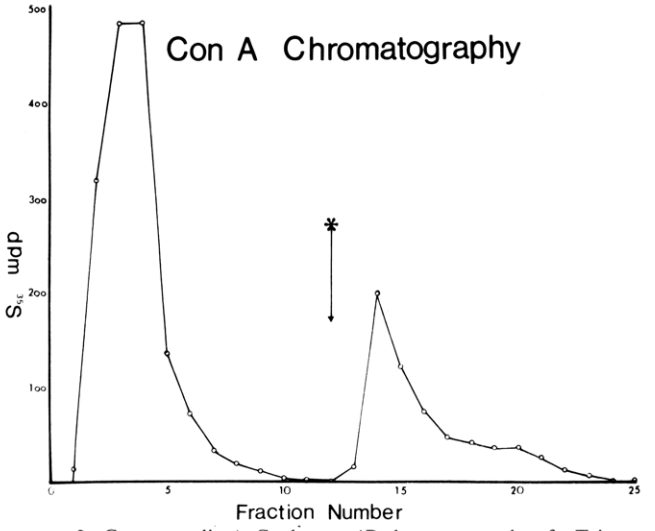

FIGURE 2: Concanavalin A–Sepharose 4B chromatography of a Triton X-100 extract of purified synaptic plasma membranes. (— o —) ^{35}S dpm; (*) addition of methyl α-D-mannoside to the elution buffer.

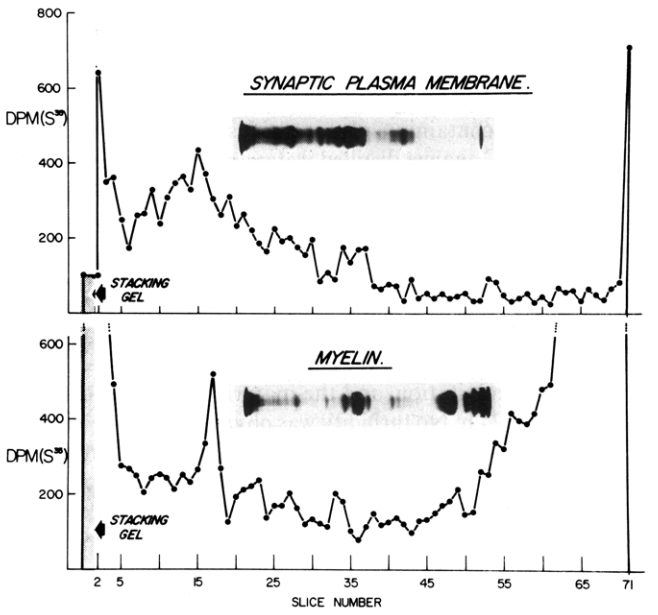

FIGURE 3: Radioactive profiles of purified synaptic plasma and myelin membrane electrophoresed on 7.5% acrylamide gels in the presence of sodium dodecyl sulfate. (— o —) ^{35}S dpm; Inserts: Photographs of Coomassie blue stained acrylamide gels.

characteristic myelin proteins. This result was confirmed by the absence of characteristic myelin-like trilaminar membrane structures in electron micrographs of the synaptic plasma membrane fraction. This suggests that the myelin contamination of synaptic plasma membranes, utilizing this method of purification, is no more than the 5% established through enzymatic assays.

Affinity Chromatography of Solubilized Synaptic Plasma Membranes on Concanavalin A–Sepharose. The solubilization procedure, previously described for synaptic plasma membrane (Waehneldt et al., 1971), was employed due to the selective extraction of periodic acid–Schiff positive material by Triton X-100. This procedure resulted in the solubilization of 44% of the ^{35}S label and approximately 65% of the protein from membranes. Affinity chromatography of the solubilized ^{35}S-labeled protein on a concanavalin A–Sepharose 4B column gave the radioactive profile seen in Figure 2. Nearly 90% of the protein and 80% of the radioactivity appeared in the first peak. The column was washed with 10 bed volumes of buffer until radioactivity and A_{280} had returned to background. Upon subsequent addition of methyl α-D-mannoside, nearly 20% of the applied radioactivity appeared in the second peak. Concentrated borate ion did not result in elution of additional radioactivity. Total recovery of radioactivity was greater than 95%. The eluted material was presumed to be sulfated glycoprotein which interacts specifically with concanavalin A.

Sodium Dodecyl Sulfate Gel Electrophoresis. Analysis of membrane fractions on 15% acrylamide gels suggested that the majority of ^{35}S-labeled protein was present at the top of gels and was not well resolved. The predominantly labeled lipid, cerebroside sulfate, could be extracted prior to electrophoresis (Greenfield et al., 1971) or resolved from membrane proteins with increasing acrylamide concentration. Since we were interested in the number and molecular weight distribution of the large sulfated glycoproteins, we subjected the membranes to electrophoresis in a standard 7.5% acrylamide gel as seen in Figure 1, C and D. When duplicate gels were stained and sliced and the radioactivity was extracted as illustrated in Figure 3, the radioactive profiles in the upper half of the gels are quite distinct and the low-molecular-weight myelin proteins are essentially absent in the synaptic plasma membrane fraction. Myelin contains a single predominant sulfated glycoprotein while the synaptic plasma membrane fraction contains a number of high-molecular-weight sulfated components. Also, the predominant sulfated component of synaptic plasma membrane has a chromatographic mobility similar to that of the major myelin glycoprotein.

In a series of double labeling experiments employing L-[^{3}H]fucose and $Na_2{}^{35}SO_4$, those high-molecular-weight components which were labeled with sulfate also were labeled

with fucose, suggesting that they were sulfated glycoproteins and not glycolipids or glycosaminoglycans. Fucolipids would be disassociated from protein in sodium dodecyl sulfate and migrate faster, and fucose has not been conclusively shown to be linked covalently to glycosaminoglycans of brain. In addition, carefully controlled staining of gels containing sodium dodecyl sulfate (Simpson et al., 1974) revealed diffuse Schiff-positive material corresponding to those regions of the gel containing ^{35}S-labeled sulfate or L-[^{3}H]fucose.

Analysis of the Sulfated Components. Electrophoresis of membranes in sodium dodecyl sulfate indicated that high-molecular-weight sulfated glycoproteins were present. The absolute identity of those sulfated macromolecules as sulfated glycoproteins and not protein associated with cerebroside sulfate or sulfated glycosaminoglycans required exhaustive lipid extraction and characterization of the ^{35}S-labeled material in lipid extracts as well as characterization of the lipid insoluble residue which may contain glycosaminoglycans as well as sulfated glycoproteins.

Lipid Extraction of Membranes. Aliquots of purified synaptic plasma and myelin membrane, equivalent to 24 mg of membrane protein, were quantitatively extracted with chloroform–methanol. The lipid-extractable counts represented 31.5% of the label in synaptic plasma membrane compared with 89.5% of the label in myelin.

Thin-Layer Chromatography and Sephadex LH-20 Chromatography of Lipid Extracts. To compare the total lipids extracted from synaptic plasma and myelin membranes, for a direct comparison of the sulfated lipid components present in each membrane fraction, equal aliquots of the lipid extract were chromatographed on silica gel G; the results are seen in Figure 4. Due to the small amount of cerebroside sulfate in the synaptic plasma membrane fraction, the iodine stain is not as apparent as in the lipid extract of myelin. However, extraction of silica gel for radioactivity indicated that 98% of the radioactivity present in lipid extracts of both synaptic plasma and myelin membrane is in cerebroside sulfate. It is important to note that nonsulfated cerebroside is essentially absent in the lipid extract of synaptic plasma membrane. The absence of nonsulfated cerebroside suggests there is no significant myelin contamination of purified synaptic plasma membrane which is consistent with the electrophoretic results seen in Figure 1. Cholesterol sulfate or the sulfolipid recently identified in whole rat brain (Flynn et al., 1975) were not detectable.

Sephadex LH-20 chromatography was also used to characterize the labeled material in lipid extracts of membranes. The resulting chromatographic profiles are essentially identical with the majority of the labeled lipid eluting with chloroform–methanol (1:1 v/v) where standard cerebroside sulfate was shown to be eluted. Again, chromatography on silica gel determined that essentially 100% of the labeled material which was resolved on Sephadex LH-20 was cerebroside sulfate.

To assess the origin of cerebroside sulfate in synaptic plasma membrane, since this lipid is reported to be absent from this neuronal membrane (Morgan et al., 1971), the specific activity of cerebroside sulfate was determined for synaptic plasma, myelin, and microsomal membranes. The specific activity of synaptic plasma membrane was nearly twice that of myelin, whereas synaptic plasma membranes and microsomes were essentially identical. Also, since the total amount of cerebroside sulfate in synaptic plasma membrane could be accounted for by a maximum of 15% contamination from microsomes, we cannot conclude that cerebroside sulfate is a membrane constituent of synaptic plasma membrane.

Membrane Specific Activities. Although 31 and 89% of the label in synaptic plasma and myelin membrane, respectively, were lipid soluble, it was necessary to characterize the lipid-insoluble material as sulfated glycoprotein and distinguish it clearly from the glycosaminoglycans known to occur in brain, although these components are not generally considered to be constituents of purified synaptic plasma membrane or myelin. Margolis et al. (1975) have recently reported the enrichment of these polymers in light and heavy microsomes from brain. To determine if microsomal contamination could account for the lipid-insoluble sulfated components of synaptic plasma membrane, specific activities were compared before and after lipid extraction of membrane fractions. The results indicate that the specific activity of synaptic plasma membrane is twice that of microsomes after lipid extraction and nearly ten times that of myelin. It is unlikely that selective contamination of synaptic plasma membrane occurs only from sulfated components of microsomes and, therefore, the high specific activity of this neuronal membrane suggests that the sulfated material in lipid-extracted membranes is not due to microsomal contamination.

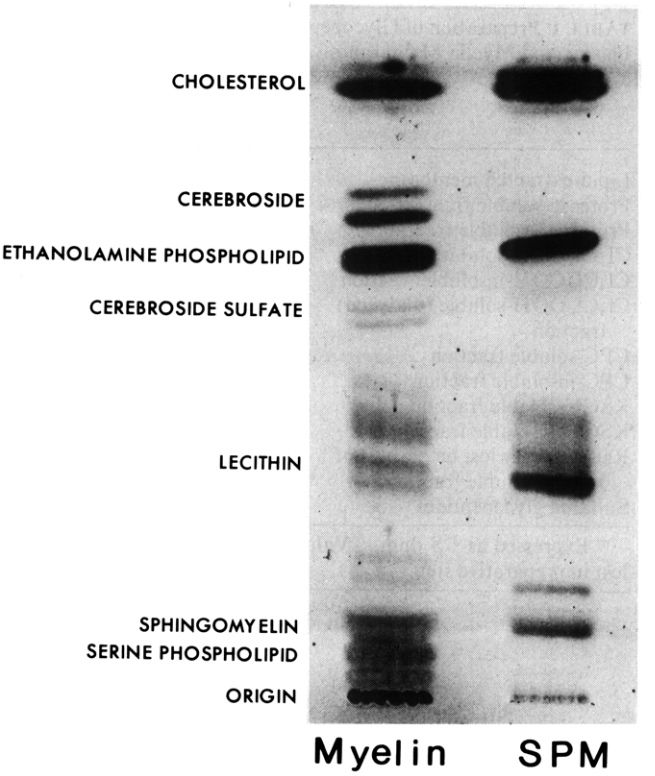

FIGURE 4: Thin-layer chromatogram of a total lipid extract of purified myelin and synaptic plasma membrane (SPM) on silica gel G showing the presence of cerebroside sulfate in synaptic plasma and myelin membranes.

Preparation of Sulfated Glycopeptides. Since the quantitative extraction of lipid grossly denatures residual membrane proteins, it was necessary to identify the high-molecular-weight sulfated glycoproteins as the products of proteolytic digestion, sulfated glycopeptides. It was also important to demonstrate that the proteolytic digest was free of sulfated glycosaminoglycans that may be specifically or adventitiously associated with purified membranes. Lipid-extracted synaptic plasma membrane and myelin, equivalent to 24 mg of membrane protein, was homogenized and extensively digested with protease as described. A summary of the recoveries of radioactivity obtained in the preparation of sulfated glycopeptides is seen in Table I. Precipitation of the protease-soluble fraction with

TABLE I: Preparation of Glycopeptides from Purified Synaptic Plasma and Myelin Membranes.

	Synaptic Plasma Membrane	Myelin
Lipid-extracted membrane	843 000[a]	160 800[a]
Protease-soluble fraction	700 000	100 400
Protease-insoluble fraction	(143 000)[b]	(60 400)[b]
Cl_3CCOOH-soluble fraction	650 000	86 600
Cl_3CCOOH-insoluble fraction	(50 000)	(14 800)
Cl_3CCOOH-soluble (dialyzed) fraction	497 900	72 400
CPC-soluble fraction	285 200	43 300
CPC-insoluble fraction	(212 700)	(29 100)
KSCN-soluble fraction	277 800	42 600
KSCN-insoluble fraction	(7 400)	(700)
Radioactivity lost on dialysis of KSCN-soluble fraction	(900)	(500)
Sulfated glycopeptides	276 900	42 100

[a] Expressed as ^{35}S dpm. [b] Values in parentheses represent label lost in preparative step.

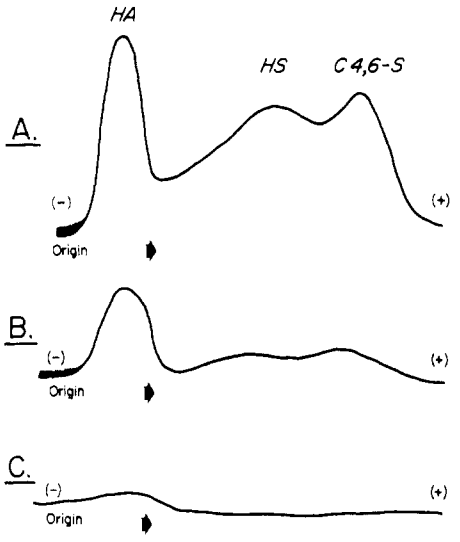

FIGURE 5: Electrophoretic profiles of (A) standards, (B) synaptic plasma membrane, and (C) myelin glycosaminoglycans recovered from purified membrane fractions and resolved on cellulose acetate. (HA) Hyaluronic acid; (HS) heparin sulfate; and (C 4,6-S) a mixture of chondroitin 4 and 6-sulfate.

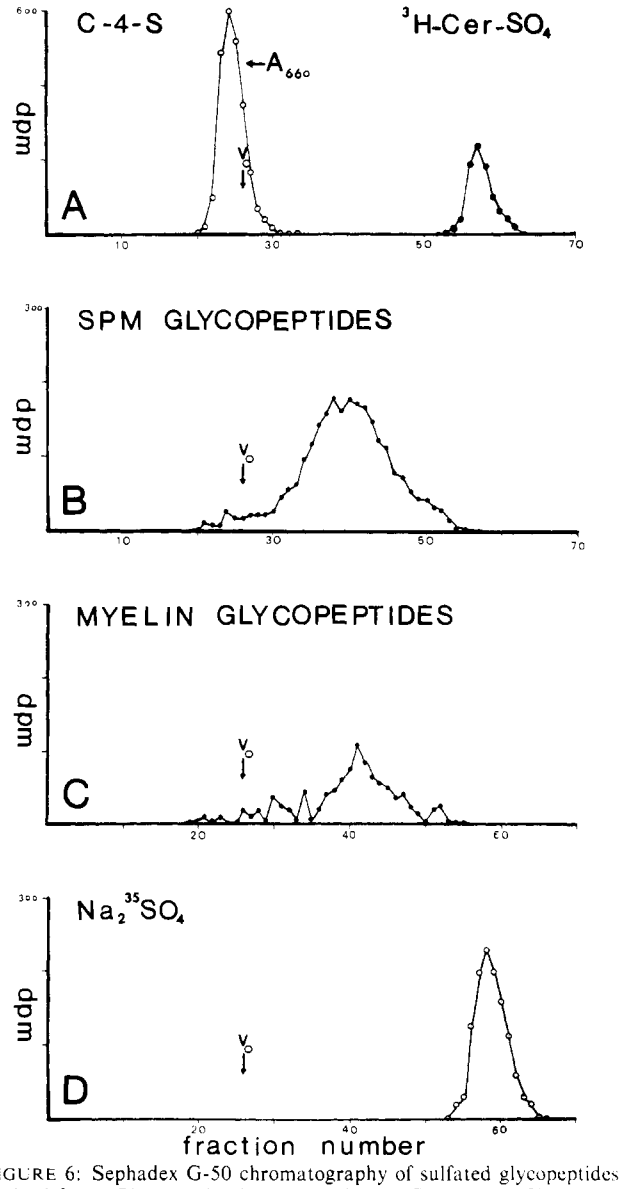

FIGURE 6: Sephadex G-50 chromatography of sulfated glycopeptides derived from (B) synaptic plasma membrane (SPM) and (C) myelin. (— O —) ^{35}S dpm. In A, we see the chromatography of chondroitin 4 and 6-sulfate (— O —) A_{660} by the orcinol assay, and the chromatography of [^{3}H]cerebroside sulfate (- - ● —) ^3H dpm. In D we see the chromatographic behavior of sodium [^{35}S]sulfate (— O --) ^{35}S dpm. V_0 indicates the void volume determined with Blue Dextran.

trichloroacetic acid resulted in a 7% loss of label in the synaptic plasma membrane fraction and 15% in the myelin, suggesting the myelin had a greater proportion of large glycopeptides. Redigestion of the protease-insoluble residue failed to solubilize a significant amount of radioactivity. The majority of the sulfated glycopeptides from whole brain were reported to be nondialyzable (Margolis and Margolis, 1970). The dialyzed fractions were adjusted to the critical electrolyte concentration for quantitative precipitation of glycosaminoglycans. Excess cetylpyridinium chloride was precipitated with KSCN and these procedures resulted in a 43 and 40% reduction of label in the synaptic plasma membrane and myelin supernatants, respectively. Supernatants were dialyzed exhaustively against deionized water and termed the sulfated glycopeptide fractions, although the glycopeptides present were heterogeneous with respect to their degree of sulfation. The recovery of label in sulfated glycopeptides following this extensive preparative procedure was 33% for synaptic plasma membrane and 26% for myelin.

Analysis of Precipitated Glycosaminoglycans. Glycosaminoglycans were converted to the sodium salts and the individual components identified by cellulose acetate electrophoresis. As seen in Figure 5, B and C, very little glycosaminoglycan was associated with synaptic plasma membrane (Figure 5B) or with myelin (Figure 5C). The material chromatographed, and, as seen in Figure 5, represents 50% of the total glycosaminoglycan associated with the purified membrane fractions. Due to the low amounts of glycosaminoglycan present in synaptic plasma membrane and myelin fractions, it is not possible to determine if their association with these membranes is specific, or due to contamination with microsomal membranes which are reported to be rich in these constituents (Margolis et al., 1975).

Sephadex G-50 Chromatography of Sulfated Glycopeptides. Aliquots of sulfated glycopeptide from synaptic plasma membrane and myelin were chromatographed on Sephadex

G-50. The results seen in Figure 6, B and C, show that no labeled glycopeptide was excluded from G-50 while continued elution gave rise to single broad symmetrical peaks of radioactivity. The recovery of radioactivity in these peaks is essentially quantitative. Since the exclusion limit, determined with polysaccharides, is 10 000 and glycopeptides behave in a similar fashion, the results suggest that the glycopeptides are somewhat heterogeneous with respect to molecular weight, and weigh less than 10 000. To compare the behavior of glycopeptides with saccharides derived from glycosaminoglycans, a mixture of pure chondroitin 4-sulfate and chondroitin 6-sulfate chains prepared by β elimination in the presence of sulfite ion (Simpson et al., 1972) and previously defined to weight 13 000 ± 1000 was chromatographed. The results seen in Figure 6A show a single sharp peak of uronic acid containing saccharide which is excluded on Sephadex G-50. The behavior of cerebroside sulfate on Sephadex G-50, also shown in Figure 6A, suggests that it is below the critical micelle concentration. The chromatographic behavior of $Na_2{}^{35}SO_4$ is shown in Figure 6D. These studies show that the somewhat heterogeneous group of sulfated glycopeptides are included on Sephadex G-50 when compared with saccharide chains derived from chondroitin sulfate, suggesting a molecular weight range of less than 10 000 compared with 13 000 or greater which one would expect from protease-treated glycosaminoglycans. Any residual free $Na_2{}^{35}SO_4$ would have been lost in the purification procedure which involved extensive dialysis of purified membranes against cold Na_2SO_4. Elimination of cerebroside sulfate as a possible contaminant was confirmed by the absence of radioactivity in chloroform–methanol extracts of glycopeptide fractions.

Acid Hydrolysis of Glycopeptides and $BaSO_4$ Precipitation. To demonstrate that radioactivity in glycopeptide fractions represented O-esterified sulfate, rather than cysteine or methionine in residual peptide fragments, acid hydrolysis and carefully controlled barium sulfate precipitation were performed. This resulted in the precipitation of 97% of the label in glycopeptide hydrolysates indicating that ^{35}S was present as inorganic sulfate. Tritiated cysteine or ^{35}S-labeled methionine, either free or in association with exogenous protein, did not precipitate under similar conditions.

Carbohydrate–Peptide Composition of the Glycopeptide Fractions. To quantitate the relative carbohydrate–peptide composition of the membrane derived glycopeptides, peptide, and neutral carbohydrate concentrations were measured. Since the average size of glycopeptides from each fraction was similar the relative percent neutral carbohydrate was calculated. The glycopeptide fraction from myelin containing 67% neutral carbohydrate was somewhat enriched in sugar compared with the fraction derived from synaptic plasma membrane consisting of 41% carbohydrate on a weight basis.

Amino Acid Analysis of Glycopeptides by Dansylation and Polyamide Chromatography. A comparison of the amino acid composition of sulfated glycopeptides obtained by hydrolysis, dansylation, and chromatography on polyamide indicates that glycopeptides from synaptic plasma membrane are particularly rich in glutamic acid (glutamine), serine, and proline with less threonine, aspartic acid (asparagine), and glycine. The myelin glycopeptides are enriched in aspartic acid (asparagine), serine, glycine, and lysine with lesser concentrations of glutamic acid (glutamine), alanine, threonine, and proline. The myelin fraction is more homogeneous than synaptic plasma membrane consistent with the number of radioactive polypeptides observed on gels. The relative patterns, although distinct, are not unique, and are generally characteristic of a glycopeptide fraction. Cysteine, cysteic acid, methionine sulfone, or tyrosine were not detectable on chromatograms.

Dowex 50 Chromatography. A partial enrichment of the sulfated glycopeptide fraction of synaptic plasma membrane was accomplished by Dowex 50 chromatography at pH 3. The chromatographic profile suggests that 98% of the counts, which represents 66% of the total peptide, did not bind to Dowex 50. At a higher ionic strength an additional 2% of the counts were eluted with 22% of the peptide. Although these results suggest that little sulfated glycopeptide bound to Dowex-50 under these conditions, the total unbound peptide, 66%, is not necessarily sulfated but may represent a proportion of sialic acid rich glycopeptide of sufficient charge density to pass, unretarded, through the column.

Mild Acid Hydrolysis of Sulfated Glycopeptides. In order to investigate the nature of the sulfate–carbohydrate linkage, glycopeptides were hydrolyzed at 100 °C in 0.25 N HCl and aliquots removed at varying periods of time. The rate of hydrolysis of axial sulfate, position 4, has been shown to be significantly greater than that of primary or equatorial sulfate esters (Rees, 1963; Meezan and Davidson, 1967). The percentage of sulfate hydrolyzed at each time period was determined by electrophoretic separation of inorganic sulfate from sulfated glycopeptide in a standardized system. When the log percent sulfate hydrolyzed was plotted as a function of time, the rate of release of labeled sulfate was found to be essentially linear, suggesting that a first-order reaction was involved. From this, the half-times of hydrolysis, determined to be 99 min, are consistent with half-times of hydrolysis determined for galactose 6-sulfate by other workers (Hirst et al., 1965; Cumar et al., 1968; Margolis and Margolis, 1970) and quite similar to the rate of glycopeptide–sulfate hydrolysis reported for galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate by Margolis and Margolis (1970).

The rate of hydrolysis of the glycopeptide fractions was linear compared with the markedly nonlinear rate for heparin sulfate (Meezan and Davidson, 1967), and the half-time of hydrolysis, 99 min, was considerably shorter than that reported for heparin sulfate, 148 min, by Margolis and Margolis (1970).

Uronic Acid Determination. No uronic acid was detectable in glycopeptide fractions when determined by the orcinol method or the borate ion modification of the carbazole assay. Paper chromatography of glycopeptide hydrolysates also failed to detect the presence of uronic acid or uronolactones. This was confirmed by the absence of glucuronic or iduronic acid on chromatograms obtained by gas–liquid chromatographic analysis of the trimethylsilyl methyl glycosides prepared from glycopeptide hydrolysates.

Carbohydrate Analysis by Gas–Liquid Chromatography. The carbohydrate composition of the glycopeptide fractions from synaptic plasma membrane and myelin was determined by gas–liquid chromatographic analysis of the trimethylsilyl methyl glycosides as described. The molar ratios of neutral sugar, hexosamine, and sialic acid in the individual glycopeptide fractions are shown in Table II.

Discussion

We have identified the presence of high-molecular-weight sulfated glycoproteins in a synaptic plasma membrane fraction from rat brain. Studies were paralleled with a myelin fraction since it was reported to contain a glycoprotein which is sulfated. A fraction of the sulfated glycoproteins from synaptic plasma membrane bound specifically to concanavalin A–Sepharose 4B, indicating that a portion of the membrane-bound receptors

TABLE II: Carbohydrate Composition of Sulfated Glycopeptides from Synaptic Plasma and Myelin Membranes.

	Molar Ratio	
	Synaptic Plasma Membrane	Myelin
Mannose	1.00[a]	1.00[a]
Fucose	0.37	0.23
Galactose	0.65	0.47
N-Acetylgalactosamine	0.00	0.00
N-Acetylglucosamine	1.10	0.52
N-Acetylneuraminic acid	0.56	0.75
Glucuronic acid	0.00	0.00
Iduronic acid	0.00	0.00
Glucose	0.34	3.80

[a] Molar ratio of monosaccharides relative to mannose in sulfated glycopeptide fractions.

for this lectin, previously demonstrated in synaptic plasma membrane (Matus et al., 1973) are sulfated. Analysis of sulfated glycoproteins by electrophoresis on acrylamide gels indicated that synaptic plasma membrane contains a number of high-molecular-weight sulfated glycoproteins compared with myelin which has a single predominant species. Synaptic plasma membrane has a similar, if not identical, sulfated glycoprotein. It is interesting to speculate that the predominant sulfated component in these membranes may be common to both glial and neuronal membranes, although cochromatography of polypeptides is less than sufficient evidence for their identity. In separate experiments, those components of synaptic plasma membrane which were labeled with sulfate, were also labeled with fucose, and were periodic acid–Schiff positive.

Since the majority of the radioactivity on gel profiles migrated with lipid, labeled membranes were quantitatively extracted and the lipid-soluble components characterized. Essentially all the radioactivity in lipid extracts of synaptic plasma membrane and myelin was associated with cerebroside sulfate and the synaptic plasma membranes fraction appeared to be enriched in the fast-moving nonhydroxylated form. The specific activities of cerebroside sulfate, determined by fractionation of crude lipid extracts, support the fact that the cerebroside sulfate present in synaptic plasma membranes is not due to myelin contamination, although the possibility that it might arise from other sources of membrane contamination cannot be ruled out.

To characterize the high-molecular-weight labeled material on gels as sulfated glycoprotein and distinguish it clearly from the glycosaminoglycans known to occur in brain, lipid-extracted membrane was digested with proteolytic enzymes and subsequently treated with cetylpyridinium chloride. Although significant radioactivity was lost on cetylpyridinium chloride and subsequent KSCN precipitation from the protease-soluble fraction, only a small fraction of the precipitated counts could be accounted for as glycosaminoglycans by electrophoresis on cellulose acetate. It is possible that a fraction of sulfated glycopeptide was of sufficient size, degree of sulfation, or sialic acid rich to be precipitated with cetylpyridinium chloride.

To compare the structures of sulfated glycopeptide derived from synaptic plasma membrane and myelin, the nondialyzable glycopeptide fractions were characterized. Hydrolysis and careful $BaSO_4$ precipitation eliminated the possibility that the residual radioactivity in glycopeptide was due to cysteine or methionine. In addition, sulfur-containing amino acids were absent on polyamide maps of dansylated glycopeptide hydrolysates. The presence of sulfate in ester linkage to oligosaccharides was supported by the kinetics and half-time of mild acid hydrolysis which indicate that the sulfate is covalently linked to a primary hydroxyl group. It is probable that sulfate is linked to the 6-hydroxyl group of galactose or *N*-acetylglucosamine, although we have not identified these sulfated monosaccharides. The structural sequence of the sulfated oligosaccharides by classical methods would require a larger amount of material than the present purification procedure affords. The presence of *N*-sulfate linkages, known to occur in heparin sulfate, was not investigated since their half-times of hydrolysis are much shorter than the value determined for the glycopeptide fraction. The absence of heparin sulfate in sulfated glycopeptide fractions was also supported by their behavior on gel filtration and the failure to detect iduronic acid or glucuronic acid by paper or gas–liquid chromatography.

Gel-filtration studies of sulfated glycopeptide fractions from synaptic plasma membrane and myelin on Sephadex G-50 show a single broad, but included, peak of radioactivity while standard glycosaminoglycans were excluded. This suggests that the glycopeptides weigh less than 10 000 but represent the larger, nondialyzable glycopeptides, with the more peripherally located monosaccharides, biosynthetically intact. The chromatographic behavior of the fractions resembled that of glycopeptides and not glycosaminoglycans.

Uronic acid was not detectable in fractions assayed by the orcinol or modified carbazole procedures. Neither glucuronic acid, iduronic acids, nor their respective lactones were detectable, when fractions were hydrolyzed under conditions giving maximum release of uronic acid and chromatographed on paper. In addition, the uronic acids were absent from chromatograms obtained by resolution of the trimethylsilyl methyl glycosides, and *N*-acetylgalactosamine, characteristic of several glycosaminoglycans, was absent.

The amino acid composition of synaptic plasma membrane and myelin glycopeptides are distinct, respectively, and are generally representative of glycopeptides prepared by proteolytic digestion. Little can be concluded about the nature of the carbohydrate–peptide linkage from these chromatograms since serine, threonine, and aspartic acid (asparagine) are well represented.

Carbohydrate analysis revealed that *N*-acetylgalactosamine was absent which suggests that the nondialyzable sulfated oligosaccharides are covalently bound through an N-glycosidic linkage between *N*-acetylglucosamine and asparagine in the polypeptide backbond. This linkage has been reported to be predominant in brain glycoproteins (Margolis and Margolis, 1973). Both synaptic plasma membrane and myelin glycopeptides are rich in mannose, consistent with the binding of sulfated glycoprotein to concanavalin A. Although glucose is not generally found as a constituent of glycoproteins and is believed to arise from various sources of contamination, it has been observed in whole brain glycopeptide fractions (Javaid et al., 1975). We are unable to account for the unusually high proportion of glucose present in the sulfated glycopeptides from myelin since the procedures employed in their preparation from both purified membrane fractions were identical and performed in parallel to eliminate minor procedural variations. The glycopeptide fraction from synaptic plasma membrane is richer in fucose than that from myelin.

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