Sulfated Glycopeptides from Rat Brain Glycoproteins*

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ABSTRACT: Glycopeptides containing hexosamine, galactose, mannose, fucose, sialic acid, and sulfate have been obtained from rat brain glycoproteins following digestion with pronase and removal of acid mucopolysaccharides by precipitation with cetylpyridinium chloride. Nonsulfated glycopeptides are also present and are similar to the sulfated glycopeptides in their molar ratios of hexosamine and neutral sugars. Fractionation of the sialic acid free glycopeptides on Dowex 1 demonstrated that approximately 40% of the hexosamine and neutral sugar is in oligosaccharide chains containing ester sulfate.

No uronic acid containing mucopolysaccharides were present in the glycopeptide fractions. Preliminary studies of the sulfated monosaccharides released by partial acid hydrolysis of the glycopeptides indicated the presence of galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate.

It has been established that sulfated and nonsulfated acid mucopolysaccharides (*i.e.*, chondroitin 4- and 6-sulfates, heparan sulfate, dermatan sulfate, and hyaluronic acid) are present in brain (Szabo and Roboz-Einstein, 1962; Margolis, 1967; Singh and Bachhawat, 1968), as well as glycoproteins containing mannose, galactose, fucose, sialic acid, glucosamine, and galactosamine, but no uronic acid (Di Benedetta *et al.*, 1969; Brunngraber *et al.*, 1969). Most tissue glycoproteins including those of brain have not been reported to contain sulfate, although several workers have described sulfated glycopeptides obtained by proteolytic enzyme digestion of gastrointestinal mucosa and certain mucous secretions (Bignardi *et al.*, 1964; Havez *et al.*, 1965; Hakkinen *et al.*, 1965; Pamer *et al.*, 1968; Nemoto and Yosizawa, 1969). We are reporting here the presence of ester sulfate in glycopeptides derived from rat brain glycoproteins. These glycopeptides appear to be distinct in structure and composition from any of the known classes of sulfated acid mucopolysaccharides.

Experimental Section and Results

Materials. β-Galactosidase from *Escherichia coli* and D-galactose dehydrogenase from *Pseudomonas fluorescens* were obtained from Boehringer-Mannheim Corp., New York, N. Y. Pronase was obtained from Calbiochem, Los Angeles, Calif., and chondroitinase ABC, chondro-6-sulfatase, and galactose 6-sulfate were from Miles Laboratories, Elkhart, Ind. Neuraminidase from *Clostridium perfringens* was obtained from Worthington Biochemical Corp., Freehold, N. J., and testicular hyaluronidase (16,000 NF units/mg) was from AB Leo, Hälsingborg, Sweden. Samples of *N*-acetylglucosamine 6-*O*-sulfate and *N*-acetylgalactosamine 6-*O*-sulfate were kindly provided by Professor K. S. Dodgson and Dr. A. H. Olavesen, and keratan sulfate by Dr. J. A. Cifonelli.

Analytical Methods. Hexosamine was measured by the manual modification of the Elson–Morgan method described by Swann and Balazs (1966), after hydrolysis of samples for 2 hr in 6 N HCl at 100°. A hydrolysis curve showed maximum release of hexosamine from brain glycopeptides at 1–2 hr under these conditions. Total neutral sugar was measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956) using a standard containing 40% galactose, 40% mannose, and 20% fucose.

Galactose was measured both by the secondary cysteine reaction of Dische (1955), and by an assay similar to that described by Lindahl and Rodén (1965), using D-galactose dehydrogenase. In the latter method, 0.2 ml of sample was added to 3.1 ml of a solution of 0.1 M Tris buffer (pH 8.6) containing 0.42 mM NAD. The reaction was started by the addition of 0.02 ml of galactose dehydrogenase solution containing 5 mg of enzyme/ml. Under the conditions of assay, 0.1 μmole of D-galactose should give an absorbance at 340 mμ of 0.188 based on a molar absorbancy of 6.22×10^3 for NADH. This value was approached in 30–40 min at room temperature; 1 μmole of D-mannose, L-fucose, D-glucose, D-glucosamine, or D-galactosamine has no effect on the oxidation of 0.1 μmole of D-galactose, and the assay was not affected by the presence of 1.0 mmole of NaCl in the reaction mixture. D-Galactose 6-sulfate was also oxidized by this enzyme, but at a rate about 25% of that of D-galactose. It was found that maximum release of galactose from brain glycopeptides occurred after 2–3-hr hydrolysis in 1 N HCl at 100°. Therefore, enzymatic galactose determinations were carried out after 3-hr hydrolysis under these conditions. The hydrolysate was neutralized with NaOH before assay. Galactose values obtained with the enzymatic and colorimetric methods agreed within 5–10%.

Methylpentose was determined by the method of Gibbons (1955). Since it has been demonstrated that fucose is the only methylpentose present in rat brain glycoproteins (Brunngraber and Brown, 1964), methylpentose values are reported in terms of fucose.

Mannose values were calculated from the difference between the total neutral sugar (using a standard of fucose,

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mannose, and galactose in the approximate proportions present in the glycopeptides) and the total of fucose and galactose determined individually using specific colorimetric or enzymatic methods. In certain glycopeptide fractions obtained by column chromatography, the mannose values thus calculated by difference were multiplied by 0.87 so as to correspond with the recovery of fucose (88%) and galactose (86%) in those fractions, assuming a similar recovery of mannose. Mannose was also determined by the cysteine–sulfuric acid reaction described by Dische and Danilchenko (1967), and gave somewhat higher values than those calculated as described above. Since in several cases the mannose values determined colorimetrically brought the total neutral sugar to greater than 100% of that determined by the phenol–sulfuric acid method, we feel that the calculated values for mannose are more accurate, although still subject to an error of 10–15%.

Hexuronic acid was measured by a borate modification of the carbazole reaction (Bitter and Muir, 1962), and sulfate was determined according to the method of Spencer (1960).

For amino acid analysis using the Beckman Model 120C amino acid analyzer, samples were hydrolyzed for 22 hr in 6 N HCl at 110°.

Electrophoresis was performed on both Whatman No. 1 and prewashed Whatman No. 3MM paper (for preparative separations), using 0.05 M sodium borate buffer (pH 9.2, buffer I) or 0.05 N acetic acid–pyridine buffer (pH 6.0, buffer II). Separations were carried out for 1 hr at 30 V/cm. Sugars were detected with alkaline silver nitrate or with aniline–hydrogen phthalate spray.

Paper chromatography was performed on Whatman No. 1 paper with the following solvent systems: A, butanol–acetic acid–water (50:12:25, v/v); B, ethyl acetate–acetic acid–water (6:3:2, v/v); C, propanol–ethyl acetate–water (7:1:2, v/v); D, ethyl acetate–pyridine–water (8:2:1, v/v); and E, ethyl acetate–pyridine–butanol–butyric acid–water (10:10:5:1:5, v/v). Sugars were detected with the same reagents as were used for paper electrophoresis.

Radioactivity was measured in a Beckman liquid scintillation counter with an efficiency of 90% for ^{35}S, or using a Baird-Atomic radiochromatogram scanner.

Isolation of Glycopeptides and Mucopolysaccharides from Brain. For isotope experiments, 13–14-day-old Sprague–Dawley rats were injected intraperitoneally with carrier-free [^{35}S]Na_2SO_4 (6–8 μCi/g) and sacrificed after 1 or 7 days. Large-scale brain extracts were made using adult Sprague–Dawley rats for those experiments involving the isolation of partial hydrolysis products of the glycoproteins.

Brains (either from single animals or large-scale preparations) were extracted with 20 volumes of chloroform–methanol (2:1, v/v), and the residue thus obtained was reextracted once with the same volume of chloroform–methanol in the reverse ratio of 1:2 to remove remaining traces of gangliosides (Suzuki, 1965). The lipid-free protein residue was dried *in vacuo* and suspended in 0.2 M boric acid–borax buffer (pH 7.8) containing pronase (0.5 mg/ml) and 0.005 M $CaCl_2$ (Narahashi and Yanagita, 1967). The brain protein was present in a concentration of 1–3% and digestion was carried out at a temperature of 55° for 24 hr, when additional pronase was added (0.5 mg/ml) and the digestion continued for a total of 72 hr. After cooling, a small amount of undigested material was precipitated by adding trichloroacetic acid to a concentration of 10%. The solution was then neutralized with NaOH and dialyzed for 1 day against running tap water and 1 day against three changes of deionized water. Insoluble protein (containing less than 2% of the total hexosamine) was removed by centrifugation, and the supernatant was made 0.04 M in NaCl. Acid mucopolysaccharides were precipitated by addition of a slight excess of CPC,[1] and hyaluronic acid was separated from the sulfated mucopolysaccharides as described previously (Margolis, 1967).

The CPC supernatant containing the glycopeptides derived from brain glycoproteins was treated with KSCN to precipitate excess CPC. After dialysis, the solution of glycopeptides was lyophilized and redissolved in 15% of the original volume of 0.04 M NaCl. Retreatment with CPC led to the formation of a new precipitate (P-2), which on the basis of preliminary data would appear to consist primarily of low molecular weight hyaluronic acid. This material was reprecipitated with ethanol and dried in the same way as the main fraction of mucopolysaccharides.

After the removal of fraction P-2, excess CPC was again precipitated with KSCN and the supernatant containing the glycopeptides was dialyzed against tap water and deionized water.

Labeled sulfate was generally used as a marker for the sulfated glycopeptides in experiments designed to further characterize this material. In young rats injected with labeled inorganic sulfate and sacrificed 1 day after injection, it was found that 30% of the total radioactivity in the dialyzed pronase digest was associated with glycopeptides derived from brain glycoproteins (including those glycopeptides which were precipitated in fraction P-2). Sulfated acid mucopolysaccharides accounted for the remaining 70% of the radioactivity in the dialyzed pronase digest. Less than 5% of the total sulfur radioactivity in the lipid-free brain protein residue was in the form of sulfur-containing amino acids. The distribution of radioactivity between mucopolysaccharides and glycoproteins was similar in rats sacrificed 1 week after injection with labeled sulfate, although it appears from our preliminary data that the glycoprotein sulfate has a somewhat more rapid turnover rate.

Comparison of the Sulfated Glycopeptides with the Acid Mucopolysaccharides. No uronic acid was found in the glycopeptides remaining after removal of fraction P-2. Although a yellow color was obtained in the carbazole reaction, this is not characteristic of uronic acid, as was confirmed by measuring the ratio of the absorbancy at 350 mμ as compared to that at 430 mμ (Mathews and Cifonelli, 1965). In the case of glucuronic acid this ratio was found to be in the range of 5–7 (depending on the concentration), whereas it was 1 or less for the glycopeptides, including the sulfated fraction isolated by ion-exchange chromatography (see below). Moreover, our experiments with labeled mucopolysaccharides also suggest that the CPC fractionation technique is highly efficient in removing mucopolysaccharides from the glycopeptides. Hyaluronic acid prepared by this method contains less than 3% of the original sulfate radioactivity of the dialyzed pronase digest, and one can therefore estimate an upper limit of 3% for the contamination of brain glycopeptides by sulfated acid mucopolysaccharides.

[1] Abbreviation used is: CPC, cetylpyridinium chloride.

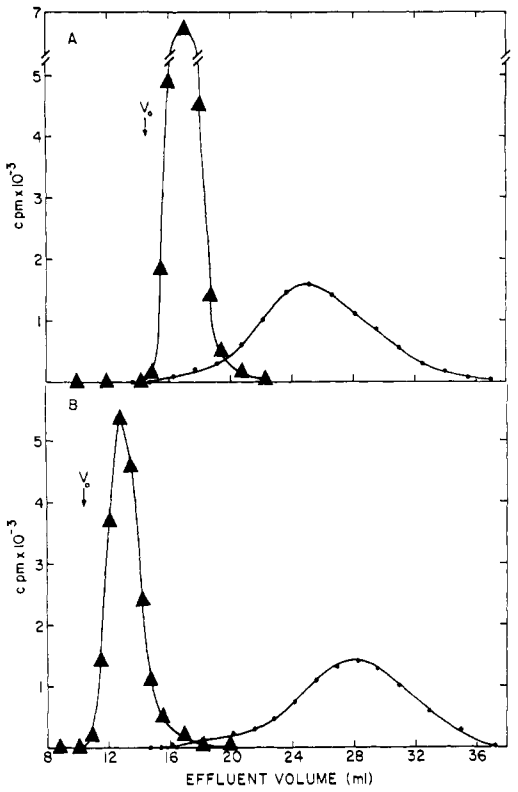

FIGURE 1: Gel filtration of ^{35}S-labeled acid mucopolysaccharides (▲—▲) and glycopeptides (●—●) on 1 × 50 cm columns of Sephadex G-50 (A) and G-75 (B), equilibrated with 0.1 M NaCl. Equal amounts of radioactivity were used for all experiments.

All of the sulfated mucopolysaccharides are excluded from Sephadex G-75, whereas the sulfated glycopeptides are eluted as a single retarded peak on a column of Sephadex G-50 (Figure 1). Since the exclusion limit (determined with polysaccharides) is 50,000 for Sephadex G-75 and 10,000 for Sephadex G-50, our data would indicate that the molecular size of the smallest sulfated mucopolysaccharides from brain is more than five times as great as that of the largest sulfated glycopeptides.

The sulfate-labeled mucopolysaccharides and glycopeptides of rat brain were also digested with chondroitinase ABC from *Proteus vulgaris* (Yamagata *et al.*, 1968). This enzyme is known to act on chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate to yield sulfated unsaturated disaccharides. It was found that when the sulfated mucopolysaccharides were treated with chondroitinase ABC and applied to a column of Sephadex G-15, approximately 25% of the radioactivity was excluded, and a single retarded peak of sulfated disaccharides accounted for the remaining radioactivity. When sulfated glycopeptides from the same brain were treated in a similar manner, no digestion was evident.

The mucopolysaccharide which is not digested by chondroitinase ABC has been identified as heparan sulfate.[2] It is known that heparan sulfate contains sulfoamino bonds which are hydrolyzed by dilute HCl or nitrous acid (Foster *et al.*, 1953; Lagunoff and Warren, 1962), as well as a varying

[2] R. U. Margolis and R. K. Margolis, unpublished results.

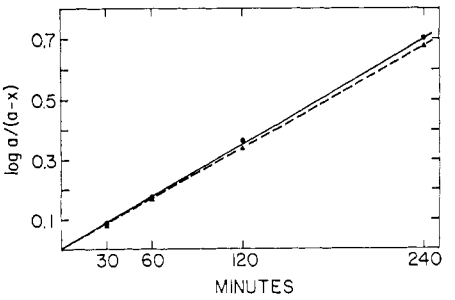

FIGURE 2: Liberation of inorganic sulfate from galactose 6-sulfate (●—●) and ^{35}S-labeled glycopeptides (△—△) during hydrolysis in 0.25 N HCl at 100°. $t_{1/2}$ = 100 min for synthetic galactose 6-sulfate and 103 min for the sulfated glycopeptides from brain.

percentage of ester sulfate which is stable under these relatively mild hydrolytic conditions (Lagunoff and Warren, 1962). The sulfated glycopeptides and heparan sulfate (obtained from a chondroitinase ABC digest of the sulfated mucopolysaccharides of brain) were treated with nitrous acid according to the procedure of Lagunoff and Warren (1962) for the determination of *N*-sulfated hexosamine. The neutralized hydrolysate contained added sodium sulfate (0.005 M) as carrier. Inorganic sulfate released by nitrous acid was separated from the remaining heparan sulfate by gel filtration on Sephadex G-25. It was found that approximately 55% of the radioactivity in brain heparan sulfate was *N*-sulfate in rats sacrificed 1 day after injection, while no *N*-sulfate was present in the sulfated glycopeptides. This would indicate that the sulfated glycopeptide fraction does not merely represent incomplete precipitation of heparan sulfate by CPC.

The sulfated glycopeptides were hydrolyzed for varying time periods in 0.25 N HCl at 100°. Hydrolyses were performed with 0.005 M sodium sulfate as carrier, and after neutralization to pH 5–6, inorganic sulfate was precipitated with barium chloride (0.025 M). The percentage of sulfate hydrolyzed was calculated from the amount of radioactivity remaining in the supernatant after centrifugation. The sulfated glycopeptides gave a linear rate of hydrolysis with a half-time of 103 min (Figure 2). This was significantly shorter than the half-time of 148 min found for the ester sulfate in heparan sulfate (Figure 3). In order to obtain a measure of the hydrolysis rate of the sulfate in brain chondroitin sulfate, the disaccharides from a chondroitinase ABC digest of the sulfated mucopolysaccharides were isolated by gel filtration on Sephadex G-15. After desalting on Sephadex G-10, treatment with chondro-6-sulfatase (Yamagata *et al.*, 1968) showed that 90% of the radioactivity was in chondroitin 6-sulfate. When the disaccharides were hydrolyzed under the conditions described above, the half-time of hydrolysis was found to be 70 min (Figure 3), which is significantly shorter than that found for the sulfated glycopeptides.

Fraction P-2 contained approximately equimolar amounts of hexosamine and uronic acid; 90% of the hexosamine was glucosamine (amino acid analyzer) and the remainder galactosamine. This material also contained small amounts (less than 10% of the hexosamine) of hexose, methylpentose, and sialic acid, in addition to 6% of the total sulfate radioactivity in the dialyzed pronase digest.

On cellulose acetate electrophoresis (Margolis, 1967) frac-

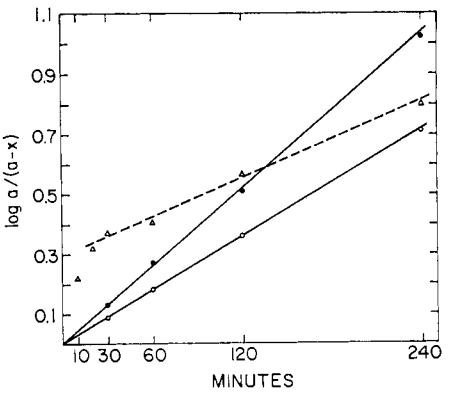

FIGURE 3: Liberation of $[^{35}S]SO_4^{2-}$ from labeled heparan sulfate (△—△), chondroitin sulfate disaccharides (●—●), and fraction P-2 (○—○) under the same conditions as in Figure 2. Heparan sulfate, fraction P-2, and the disaccharides from chondroitin sulfate were prepared from rat brain as described in the text; 90% of the sulfate in the mixed disaccharides was in the form of *N*-acetylgalactosamine 6-sulfate. $t_{1/2}$ = 148 min for the ester sulfate in heparan sulfate, 70 min for the sulfated disaccharides, and 100 min for P-2.

tion P-2 migrated as two spots. The major spot had a mobility slightly faster than that of hyaluronic acid from bovine brain (with a molecular weight of approximately 140,000). A faint, rapidly migrating spot was found in the same position as that occupied by the glycopeptides which remained after removal of fraction P-2, and which showed only a faint or no staining with acridine orange in the region of hyaluronic acid.

In 0.25 N HCl at 100° the sulfate in this fraction had the same half-time of hydrolysis as that of the sulfated glycopeptides which were not precipitated with CPC. No *N*-sulfate was present. The radioactivity in fraction P-2 was also eluted in the same position as the sulfated glycopeptides on columns of Sephadex G-25, G-50, and G-75. After treatment with testicular hyaluronidase or chondroitinase ABC the labeled material in fraction P-2 was not digested to lower molecular weight fragments as determined by gel filtration on Sephadex G-25.

On the basis of the analytical data and the identical behavior of the labeled portion of fraction P-2 with the remaining sulfated glycopeptides, we have tentatively concluded that this fraction consists primarily of low molecular weight hyaluronic acid that is not precipitated in the initial treatment with CPC. The associated sulfate, hexose, methylpentose, and sialic acid would appear to represent a small portion of the sulfated glycopeptides which are precipitated by CPC after concentration of the original solution. However, P-2 may contain more than one population of sulfated glycopeptides, possibly including one with a greater than average degree of sulfation. Further experiments to examine these various possibilities are currently in progress.

Separation of Sulfated and Nonsulfated Glycopeptides. In an attempt to determine what percentage of the carbohydrate in rat brain glycoproteins is associated with sulfate, the sialic acid free glycopeptides were chromatographed on Dowex 1 (formate). Brunngraber[3] had previously found that a portion of sialic acid free rat brain glycopeptide, when applied to an anion-exchange column, was not recovered as expected in the water effluent. In the light of our findings it appeared that the material retained on the column might represent oligosaccharide chains bearing sulfate groups.

Sialic acid was removed from sulfate-labeled rat brain glycopeptides either with neuraminidase from *Clostridium perfringens* (Cassidy *et al.*, 1965) or by hydrolysis for 1 hr in 0.1 N H_2SO_4 at 80°. Both procedures removed all of the sialic acid, but no sulfate. An aliquot of sialic acid free glycopeptide containing approximately 2.3 mg of neutral sugar, 1.5 mg of hexosamine, and 250,000 cpm of ^{35}S was applied to a 1 × 50 cm column of Dowex 1-X8, 200–400 mesh (formate form). Elution was begun with water at a rate of 5 ml/hr, and two fractions (IA and IB) of 150 ml each were collected. The column was then washed with 5 M formic acid, and four fractions were collected (IIA, 190 ml; IIB, 180 ml; IIC, 135 ml; and IID, 135/ml). 61% of the neutral sugar and 64% of the hexosamine was recovered in fraction IA, with most of the remainder in fraction IIA. Only traces of carbohydrate were found in the other fractions.

Six per cent of the applied radioactivity was not retained on the column and appeared in the water effluent (fraction IA), while an additional 45% was eluted with 5 M formic acid. However, it was found in this experiment and in two succeeding ones that only 50–60% of the radioactivity could be recovered from the column after elution with 5 M formic acid. This recovery could be increased to 60 to 70% if the formic acid was followed by 3 N HCl, although no carbohydrate was eluted in these fractions.

In order to obtain information as to whether the retained radioactivity might be acid-labile inorganic sulfate, a sample of sulfate-labeled glycopeptide was dissolved in 5 M formic acid and left for 7 days at room temperature. Gel filtration on Sephadex G-25 revealed that no sulfate was removed by this treatment. In another experiment, $[^{35}S]Na_2SO_4$ and unlabeled synthetic galactose 6-sulfate were applied to a column of Dowex 1 (formate) and eluted with water and 5 M formic acid as described above. Less than 5% of the applied radioactivity or galactose was recovered from the column, indicating that neither inorganic sulfate nor galactose 6-sulfate can be eluted from Dowex 1 with 5 M formic acid. The failure to recover galactose also demonstrates that galactose 6-sulfate is not hydrolyzed under these conditions.

Although Ryan *et al.* (1965) have reported that galactose 6-sulfate can be eluted from Dowex 1 (formate) with 4.5 M formic acid, they do not specify the cross-linkage or mesh size of the resin used. However, other workers have found that varying proportions of sulfated carbohydrates become irreversibly bound to ion-exchange materials (Love and Percival, 1964; How *et al.*, 1969). In the case of sulfated acid mucopolysaccharides, it was reported that the overall recovery of radioactivity from an anion-exchange resin was 35–45%, and that the fractionation patterns obtained with different batches of nominally identical resin varied considerably (How *et al.*, 1969). This was attributed to trapping of anions in "cracks" in the resin beads (How *et al.*, 1969). It would appear that such a process may account for the incomplete recoveries of sulfate found in our experiments.

From the analysis of the glycopeptides appearing in the water effluent and the formic acid eluate (Table I), it can be seen that approximately 40% of the carbohydrate is associated with oligosaccharide chains which are retained by Dowex 1, presumably because of the presence of sulfate

[3] E. G. Brunngraber, personal communication.

TABLE I: Chromatography of Sialic Acid Free Glycopeptides on Dowex 1 (Formate).

	Sample Applied to Column		Fraction IA (Water Effluent)		Fraction IIA (5 M Formic Acid Eluate)	
	μg Total	Molar Ratio	μg Total	Molar Ratio	μg Total	Molar Ratio
Hexosamine	1510	1.00	967	1.00	534	1.00
Neutral sugar[a]	2333		1425		1020	
Fucose	381	0.28	216	0.25	120	0.25
Galactose[b]	1265	0.84	708	0.73	384	0.72
Mannose[c]	687	0.46	295	0.31	303	0.57
Sulfate	167	0.21			157[d]	0.55
Hexuronic acid	240[e]		135[e]		150[e]	

[a] Phenol–sulfuric acid method. [b] D-Galactose dehydrogenase assay. [c] Calculated from the difference: total neutral sugar − (fucose + galactose), assuming that 87% of the mannose was also recovered in fractions IA + IIA. [d] Calculated on the basis of 94% of the sulfate being associated with this fraction. [e] Anomalous yellow color, not characteristic of hexuronic acid.

groups. However, we have studied only the larger, nondialyzable glycopeptides, which are considered to be most representative of biosynthetically complete oligosaccharide chains in the glycoproteins from which they are derived. Insofar as the dialyzable glycopeptides may be more or less highly sulfated than those which are retained after dialysis, the average degree of sulfation of rat brain glycoproteins may also be somewhat different from that of the larger glycopeptides studied here.

The sulfated glycopeptides (fraction IIA) showed no difference in the molar ratios of hexosamine, galactose, and fucose as compared to the nonsulfated glycopeptides, although they do appear to contain more mannose. It can be calculated that approximately one sulfate is present for each two molecules of hexosamine, assuming that all but 6% of the sulfate applied to the column was originally associated with the glycopeptides which are eluted with formic acid. However, precise molar ratios cannot be obtained from these data because a small amount (12–14%) of the fucose and galactose was not recovered from the column.

Certain differences were found in the amino acid composition of the sulfated and nonsulfated glycopeptides (Table II). The sulfated glycopeptides contained nearly three times as much glutamic acid and half as much threonine as the nonsulfated fraction. The percentage of the total hexosamine represented by galactosamine was also twice as high (17%) in the sulfated glycopeptides as compared to the nonsulfated glycopeptides (8%). The overall recovery of amino acids from the Dowex–formate column was approximately 95%.

Characterization of the Sulfated Monosaccharides. In order to further characterize the sulfated glycopeptides, it was of interest to isolate and identify their constituent sulfated monosaccharide(s).

Since the half-time of hydrolysis of the glycopeptide sulfate (103 min in 0.25 N HCl at 100°) was found to be very similar to the half-times of 97 and 101 min reported by other workers (Hirst *et al.*, 1965; Cumar *et al.*, 1968) for galactose 6-sulfate, and to our own figure of 100 min as determined with synthetic galactose 6-sulfate, we attempted to isolate this compound by treatment of the labeled, sialic acid free glycopeptides with β-galactosidase from *E. coli*. The glycopeptides were incubated for 20 hr at 37° with 0.25 mg of β-galactosidase in 0.05 M phosphate buffer (pH 7.0) containing 0.01 M $MgSO_4$. The incubation mixture was then applied to a short charcoal–Celite column (Darco G-60–Celite 535, 1:1, w/w) (Whistler and Durso, 1950). No radioactivity and only 7% of the total

TABLE II: Amino Acid Composition of Rat Brain Glycopeptides.[a]

	Unfractionated Glycopeptides	Nonsulfated Glycopeptides (IA)	Sulfated Glycopeptides (IIA)
	Residues/100 Residues of Amino Acid		
Lysine	2.9	3.8	1.9
Histidine	Trace	Trace	Trace
Arginine	Trace	Trace	Trace
Aspartic acid	16.9	19.0	16.5
Threonine	12.8	18.0	9.7
Serine	10.7	11.8	9.2
Glutamic acid	16.3	9.6	26.2
Proline	14.4	11.3	10.3
Glycine	8.8	6.7	10.3
Alanine	6.3	8.8	5.3
Cystine			
Valine	5.4	5.6	3.5
Methionine	Trace	Trace	Trace
Isoleucine	1.7	1.5	2.0
Leucine	2.8	2.6	1.8
Tyrosine	Trace	Trace	Trace
Phenylalanine	Trace	Trace	2.6
Glucosamine[b]	60.3	102.2	29.1
Galactosamine[b]	7.2	9.3	5.8

[a] Glycopeptides were fractionated on Dowex 1 (formate) as described under Experimental Section. Values given in the table are the averages of duplicate determinations. [b] Corrected for destruction during hydrolysis (6 N HCl, 22 hr, 110°).

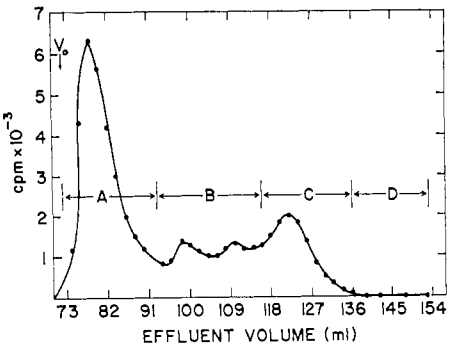

FIGURE 4: Hydrolysis of ^{35}S-labeled glycopeptide in 0.1 M HCl was carried out as described under Experimental Section. Hydrolysis products were fractionated on a 2 × 60 cm column of Sephadex G-15, equilibrated with 0.1 M NaCl.

glycopeptide galactose (determined with D-galactose dehydrogenase) was recovered in the effluent and 4% ethanol eluate. In another experiment it was found that while 5% of the glycopeptide galactose was released by the β-galactosidase from *E. coli*, this same enzyme preparation completely hydrolyzed a considerably greater amount of lactose. These findings indicate that most of the galactose in sialic acid free rat brain glycopeptides is not susceptible to β-galactosidase from *E. coli*, although it does not exclude the presence of β-galactosidic linkages, since it has been reported that the galactose in α_1-acid glycoprotein of plasma is hydrolyzed by a β-galactosidase from *Diplococcus pneumoniae* while β-galactosidase from *E. coli* has no effect (Hughes and Jeanloz, 1964). It is also possible that very little of the galactose in rat brain glycoproteins occupies a terminal position on the oligosaccharide chains, or is penultimate to sialic acid.

Sulfated monosaccharides were therefore obtained by partial acid hydrolysis. Labeled glycopeptide containing 4 μCi of ^{35}S was mixed with unlabeled glycopeptide prepared from 300 g of rat brain to give a total of 60 mg of hexosamine (90% glucosamine). Sialic acid was removed by hydrolysis in 0.1 N H_2SO_4 for 1 hr at 80°, and the sialic acid free glycopeptides were recovered by gel filtration on a 2 × 60 cm column of Sephadex G-15. The glycopeptides were then dialyzed, lyophillized, and redissolved in 5 ml of 0.1 N HCl. After hydrolysis for 90 min at 100° in the presence of 50 μmoles of Na_2SO_4 and neutralization to pH 6 with NaOH, inorganic sulfate was precipitated with $BaCl_2$ (250 μmoles), and accounted for approximately 20% of the radioactivity in the hydrolysate. The supernatant was applied to a 2 × 60 cm column of Sephadex G-15 and the effluent was pooled into four fractions (Figure 4). Approximately 57% of the radioactivity was found in a large excluded peak (fraction A), while 22% was eluted in a single peak having an elution volume corresponding to a sulfated monosaccharide (fraction C). This material was desalted on Sephadex G-10, concentrated on a rotary evaporator, and used for paper chromatography and electrophoresis.

On electrophoresis in buffer II, fraction C migrated as a single spot. Electrophoresis in buffer I resolved two components containing approximately equal amounts of radioactivity. The more rapidly migrating material had the same mobility as galactose 6-sulfate, while the other labeled sugar moved like *N*-acetylglucosamine 6-sulfate and *N*-acetylgalactosamine 6-sulfate, which were not separated.

Paper chromatography in solvents A, B, C, and D also separated two radioactive components from fraction C. In all cases, the sugar with the lower mobility migrated like galactose 6-sulfate, and the more rapidly moving sugar migrated like *N*-acetylglucosamine 6-sulfate and *N*-acetylgalactosamine 6-sulfate, which were not separated in any of these chromatographic systems. In both paper chromatography and electrophoresis, the radioactivity coincided with the spots revealed by the alkaline silver nitrate reagent. No unsulfated sugars were present.

Fraction C was separated by preparative paper electrophoresis in buffer I into two radioactive bands which were localized by autoradiography using Kodak No-Screen X-Ray film. The bands were cut out and eluted with water. The more rapidly migrating material was labeled f and the slow component was labeled s. After hydrolysis for 6 hr in 1 N HCl at 100° and neutralization with Dowex 3 (CO_3^{2-}), electrophoresis in buffer I and paper chromatography in solvents B, D, and E showed that f contained only galactose. The material (s) which migrated on electrophoresis like an *N*-acetylhexosamine 6-sulfate yielded only glucosamine after hydrolysis followed by paper chromatography and electrophoresis in the same manner as described above for f.

Fraction B from the 0.1 N HCl hydrolysate of the glycopeptides was isolated by gel filtration on Sephadex G-15 and desalted on Sephadex G-10 as described for fraction C. This material was then separated by electrophoresis in buffer I into two labeled fractions having the mobilities of a disaccharide and a trisaccharide, which were then hydrolyzed for 6 hr in 1 N HCl at 100°. Electrophoresis in buffer I and paper chromatography in solvents A, B, D, and E showed that the disaccharide contained galactose and glucosamine, while the trisaccharide yielded galactose, glucosamine, and mannose.

Discussion

Our findings provide evidence for the presence in rat brain of a class of sulfated glycoproteins distinct in structure and composition from the usual sulfated acid mucopolysaccharides. The failure of appropriate bacterial mucopolysaccharidases and testicular hyaluronidase to digest these sulfated glycopeptides excludes chondroitin 4- and 6-sulfates and dermatan sulfate, and the absence of sulfoamino groups as determined by nitrous acid treatment excludes heparan sulfate and heparin. The relatively large amounts of hexose and methylpentose in the sulfated fraction, as well as the absence of uronic acid, support the view that these glycopeptides are derived from brain glycoproteins rather than from sulfated acid mucopolysacharides. The only other sulfated acid mucopolysaccharide known to occur in mammalian tissues is keratan sulfate. Although keratan sulfate contains no uronic acid, it is usually included in the group of acid mucopolysaccharides because of its predominantly linear polymeric structure, containing disaccharide repeating units of galactose and *N*-acetylglucosamine, with sulfate located at the 6 position on either or both of the carbohydrate moieties (Bhavanandan and Meyer, 1967, 1968).

Our data indicate that the sulfated glycopeptides prepared from brain also contain galactose 6-sulfate and *N*-acetylglu-

cosamine 6-sulfate, but they appear to be derived from glycoproteins rather than representing a form of keratan sulfate. Among the main distinguishing features are the relatively high molar ratios of fucose and mannose to hexosamine (0.25 and 0.57, respectively) in the sulfated glycopeptides from brain, whereas the corresponding figures reported for keratan sulfate range from 0.02 or less to 0.1, depending on the tissue and species studied (Mathews and Cifonelli, 1965; Bhavanandan and Meyer, 1967, 1968; Baker *et al.*, 1969; Balduini *et al.*, 1968). Moreover, the molar ratio of sulfate to hexosamine in keratan sulfate is generally greater than 1.0, whereas in the sulfated glycopeptides from brain it is approximately 0.5, and the molar ratio of galactose to hexosamine is considerably less than 1.0, indicating that these glycopeptides are not predominantly composed of galactosyl-*N*-acetylglucosamine repeating units. The isolation in approximately equal amounts of a sulfated trisaccharide containing mannose, galactose, and glucosamine, as well as a sulfated disaccharide containing galactose and glucosamine, would indicate that a large proportion of the more peripherally located sulfated sugars are present in a trisaccharide unit containing mannose. Although there is some variation in the composition of keratan sulfate from different sources, our data lead us to conclude that the sulfated glycopeptides described here are derived from sulfated oligosaccharide chains in brain glycoproteins, and that they differ from the nonsulfated oligosaccharides only insofar as they contain sulfate and possibly additional mannose.

We have found that the sulfate in rat brain glycoproteins is located on galactose and glucosamine residues. The half-time of hydrolysis is consistent with that reported for ester sulfate on a primary hydroxyl group (Rees, 1963), and is within the range of values found by ourselves and other workers for synthetic galactose 6-sulfate. The finding that all points of the hydrolysis curve of the sulfated glycopeptides fall on a straight line would suggest that only one sulfated sugar is present. However, by paper chromatography it was possible to separate two sulfated monosaccharides. These have been tentatively identified as galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate on the basis of the chromatographic and electrophoretic behavior of the sulfated sugars and of the nonsulfated monosaccharides released by further acid hydrolysis. From these data it is necessary to conclude that the sulfated primary hydroxyl group of glucosamine 6-sulfate has the same half-time of hydrolysis as the sulfate in galactose 6-sulfate. A firm identification of the position of the sulfate on galactose and glucosamine will require additional analyses such as periodate oxidation and methylation studies, for which it will be necessary to isolate considerably larger amounts of the sulfated monosaccharides from brain.

Since keratan sulfate is also known to contain both *N*-acetylglucosamine 6-sulfate and galactose 6-sulfate in varying amounts (Bhavanandan and Meyer, 1967, 1968), a sample of keratan sulfate from bovine nasal septum was hydrolyzed under the same conditions as used for the sulfated glycopeptides. An apparent first-order reaction could be interpreted as indicating that both galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate have the same half-time of hydrolysis. It was found that the release of sulfate from keratan sulfate did give a straight-line graph but proceeded very slowly in 0.25 N HCl at 100°. From these data it appears that the sulfate in keratan sulfate is unusually stable to hydrolysis in dilute acid, especially when compared to free galactose 6-sulfate or to *N*-acetylgalactosamine 6-sulfate in chondroitin 6-sulfate (Rees, 1963). Bhavanandan and Meyer (1967) have reported a significant yield of sulfated monosaccharides and oligosaccharides from keratan sulfate after hydrolysis for 1 hr in 0.5 N H_2SO_4 at 100°, again indicating that the ester sulfate groups in the intact polysaccharide are relatively resistant to acid hydrolysis. In this respect also, the sulfated glycopeptides from brain differ from keratan sulfate.

Acknowledgments

We thank Professor K. S. Dodgson and Dr. A. H. Olavesen for providing authentic samples of *N*-acetylhexosamine 6-sulfates, and Dr. J. A. Cifonelli for samples of keratan sulfate.

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Synthesis of Cellulose Derivatives Containing the Dihydroxyboryl Group and a Study of Their Capacity to Form Specific Complexes with Sugars and Nucleic Acid Components*

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ABSTRACT: Carboxymethylcellulose can be converted into *N*-(*m*-dihydroxyborylphenyl)carbamylmethylcellulose by reaction of its azide with an aqueous solution of *m*-aminobenzeneboronic acid, and aminoethylcellulose reacts with an aqueous solution of *N*-(*m*-dihydroxyborylphenyl)succinamic acid in the presence of *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate to yield *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose. These two cellulose derivatives have been shown to form specific complexes of variable stabilities with nucleic acid components, sugars, and other polyols. In chromatography on columns prepared from these celluloses the retention volume of a particular polyol depends on (i) the availability in the compound of a glycol group with the appropriate configuration and conformation, (ii) the pH of the elution solvent, (iii) the ionic strength and the nature of the cations in the elution solvent, and, in the case of nucleosides, (iv) the nature of the base attached to the glycol group. The application of these observations to studies on the fine structure of nucleic acids is indicated.

The unsubstituted 2',3'-diol groups which are located at the 3' terminals of RNA molecules and polyribonucleotides possess some unique properties and some of these have already been exploited in structural studies on nucleic acids (Gilham, 1970a). In a recent example of one of these studies a specific reaction exhibited by this diol group is used to effect the direct separation of ribonucleic acid fragments containing the group from those fragments in which one of the terminal hydroxyl groups is substituted with a phosphate group. In this procedure, the diol group of the polynucleotide is specifically oxidized with periodate and the resulting dialdehyde is selectively bound to aminoethylcellulose from which it can be subsequently recovered (Lee *et al.*, 1970). This oxidation reaction can also be used to bind RNA molecules and polyribonucleotides permanently to cellulose, and, in this case, the initial complex formed between the oxidized polynucleotide and the amino groups of aminoethylcellulose is stabilized by reduction with sodium borohydride (Gilham, 1970b).

Another specific property exhibited by the glycol groups of nucleic acid components is that of complex formation with the borate anion and the use of these complexes in the chromatographic and electrophoretic separation of these components has been reviewed (Khym, 1967). The basis of these separations rests on the change in physical characteristics exhibited by the diol in the presence of borate and, in particular, on the extra negative charge attained by the diol in its complexed form. Although these effects have been exploited successfully in the case of nucleosides and mononucleotides the extension of the method to the study of larger molecules, *e.g.*, polynucleotides, may not be too fruitful since the percentage change in physical properties and total negative charge would decrease with increase in chain length of the polymer. In initiating the present work it seemed that the study and exploitation of this type of complex formation with both small and large molecules containing the glycol group could be readily carried out by immobilizing the dihydroxyboryl group through its attachment to an insoluble polymer.

* From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907. *Received June 22, 1970.* Supported by Grant GM 11518 from the National Institutes of Health and Grant GB 7864 from the National Science Foundation.