THE CARBOHYDRATE COMPOSITION OF BRAIN MICROTUBULE PROTEIN

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SUMMARY: Brain microtubule protein was found to contain 1.3% carbohydrate, consisting of glucosamine, galactosamine, galactose, mannose, fucose and sialic acid. Alkaline borohydride treatment destroyed 40% of the galactosamine and led to the formation of an equimolar amount of galactosaminitol, indicating the presence of alkali-labile O-glycosidic linkages of N-acetylgalactosamine to serine and/or threonine residues. Since a portion of the carbohydrate is attached to protein by alkali-stable linkages, at least two types of oligosaccharide are present, and can be calculated to contain seven monosaccharide residues per mole of microtubule protein dimer.

Microtubules are the most widely distributed type of intracellular filament, occurring in a great variety of cell types including those of plants, animals and protozoa. The microtubule subunit protein, tubulin, has been isolated from brain, sperm flagella, cilia, mitotic apparatus and other sources, and found to be a dimer with a molecular weight of 110,000-120,000 which can be dissociated into monomers of about equal weight (1-4). It has also been reported that the protein from the sea urchin mitotic spindle (5) and brain microtubule protein (6) contains small amounts of unidentified carbohydrate, but no detectable lipid. Since microtubule protein can be obtained from brain in a highly purified form and in quantities sufficient for study of the relatively small amount of carbohydrate present, we have investigated further the carbohydrate composition of this glycoprotein of nervous tissue.

MATERIALS AND METHODS: Microtubule protein was prepared from porcine brain by the batch method of Weisenberg *et al.* (4), and was found to contain only two

protein components which were identified as the tubulin subunits by high resolution polyacrylamide gel electrophoresis (7). After dialysis and lyophilization, the resulting preparation was only slightly soluble in water or dilute acid.

Hexosamines and hexosaminitols were differentially determined on a Beckman Model 120C amino acid analyzer using a 0.35 M sodium citrate buffer, pH 5.06, containing 0.3 M boric acid (8). For alkaline borohydride treatment, samples were treated for 16 hours at 45° with 0.02 N NaOH containing 0.4 M sodium borohydride (9), acidified with acetic acid, and lyophilized. Boric acid was removed as trimethylborate by repeated additions of 1:1000 HCl-methanol and evaporation in vacuo. Treated and untreated samples were hydrolyzed for 8 hours in 4 N HCl at 100°, evaporated to dryness on a rotary evaporator, and redissolved in 0.02 N HCl for application to the amino acid analyzer.

For the determination of neutral sugars, 70 mg of microtubule protein was suspended in 1 N HCl (2 ml) and hydrolyzed for 6 hours at 100°. After hydrolysis the undissolved protein residue was removed by centrifugation, and the supernatant was combined with three water washes of the residue. The combined supernatants were diluted to a concentration of less than 0.1 N HCl and passed through a mixed-bed ion exchange column containing Dowex 50-X4 (H^+) and Dowex 1-X8 (formate) to remove peptides, amino acids and hexosamines. The neutral sugars were eluted with water and concentrated on a rotary evaporator. Hydrolysis for 3 hours in 1 N HCl at 100°, or for 2 hours in 2 N trifluoroacetic acid at 120° (in which the microtubule protein dissolved completely) gave essentially the same neutral sugar values as those obtained using the hydrolysis conditions described above. Paper chromatography of the neutral sugar fraction was performed in propanol-ethyl acetate-water (7:1:2, v/v); ethyl acetate-pyridine-water (8:2:1, v/v); and ethyl acetate-acetic acid-water (6:3:2, v/v). Sugars on paper chromatograms were detected with alkaline silver nitrate. Quantitative determinations of galactose (using D-galactose dehydro-

genase) and fucose were carried out as described previously (10). Mannose and glucose were determined enzymatically as described by Finch et al. (11).

For neuraminidase treatment, 24 mg of microtubule protein was suspended in 5 ml of 0.05 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl, 5 mM $CaCl_2$, and 36 µg (550 units) of Vibrio cholerae neuraminidase (Behringwerke AG., Marburg/Lahn). Incubation was carried out for 48 h at 37° after addition of 1% toluene. The microtubule protein was then dialyzed and lyophilized for polyacrylamide gel electrophoresis and determination of residual sialic acid. The sialic acid content of microtubule protein was determined by the thiobarbituric acid method of Warren (12) and by the periodate-resorcinol method of Jourdian et al. (13) after hydrolysis of samples for 1 h in 0.1 N H_2SO_4 at 80°.

RESULTS AND DISCUSSION: Paper chromatography of the neutral sugar fraction of acid hydrolysates of brain microtubule protein demonstrated the presence of three sugars with the same mobilities as standards of galactose, mannose and fucose in the solvent systems listed above. The results of the quantitative determinations of neutral sugars, hexosamines and sialic acid are given in Table I. Although traces (< 0.2 µmoles/100 mg) of material reacting in the enzymatic assay for glucose were also detected, this small amount of glucose may have originated in the DEAE-Sephadex used in the preparation of the microtubule protein.

Alkaline borohydride treatment was carried out under conditions which cleave O-glycosidic linkages of sugars to 3-hydroxy amino acids (usually serine or threonine) in glycoproteins by a base-catalyzed β-elimination reaction (14,15), with concomitant reduction of the O-glycosyl leaving group to the corresponding alcohol. It was found that 40% of the galactosamine in brain microtubule protein was destroyed by alkaline borohydride treatment, and that an equimolar amount of galactosaminitol was formed. Although the expected destruction of a small percentage of the serine and/or threonine could not be

TABLE I

Carbohydrate Composition of Microtubule Protein

	Percent of moisture-free weight	Moles/mole of dimer*
Galactose	0.21	1.28
Mannose	0.17	1.05
Fucose	0.09	0.61
N-acetylglucosamine	0.33	1.66
N-acetylgalactosamine	0.24	1.21
N-acetylneuraminic acid	0.26	0.93
	1.30	6.74

*Based on a dimer molecular weight of 110,000.

studied in our samples due to their relatively high amino acid content (over 98%), our data indicate the presence of alkali-labile O-glycosidic linkages of N-acetylgalactosamine to serine or threonine residues in brain microtubule protein. Since microtubule protein still contained carbohydrate after alkali treatment (0.2 N NaOH, 48 hrs, 25°) and gel filtration on Sephadex G-100, it is evident that alkali-stable oligosaccharides are also present.

It would appear that both of the subunits are glycoproteins, since microtubule protein prepared from mouse brain by vinblastine precipitation and labeled with [1-^{14}C]glucosamine was found to contain approximately equal amounts of radioactivity in both subunits after their separation by polyacrylamide gel electrophoresis (16). Treatment of the microtubule protein with neuraminidase removed over 90% of the sialic acid but did not change the electrophoretic mobility of the tubulin subunits, indicating that the charged sialic acid residues are not responsible for the different mobilities of the two monomers.

Our data are consistent with the presence of at least two oligosaccharide

chains containing a total of seven monosaccharide residues per microtubule protein dimer. The nonintegral molar ratios of the component monosaccharides per mole of microtubule protein can be mainly ascribed to the known microheterogeneity of oligosaccharides in glycoproteins, although a certain degree of error is also inherent in the carbohydrate analysis of a glycoprotein containing only 0.1 to 0.3% of each individual sugar species. In order to obtain more detailed information on the carbohydrate structure of brain microtubule protein, studies are now in progress using glycopeptides prepared from considerably larger amounts of the purified glycoprotein than were available for this investigation.

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