Distribution and Metabolism of Glycoproteins and Glycosaminoglycans in Subcellular Fractions of Brain†

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ABSTRACT: The distribution, carbohydrate composition, and metabolism of glycoproteins have been studied in mitochondria, microsomes, axons, and whole rat brain, as well as in various synaptosomal subfractions, including the soluble protein, mitochondria, and synaptic membranes. Approximately 90% of the brain glycoproteins occur in the particulate fraction, and they are present in particularly high amounts in synaptic and microsomal membranes, where the concentration of glycoprotein carbohydrate is 2–3% of the lipid-free dry weight. Treatment of purified synaptic membranes with 0.2% Triton X-100 extracted 70% of the glycoprotein carbohydrate but only 35% of the lipid-free protein residue, and the resulting synaptic membrane subfractions differed significantly in carbohydrate composition. The glycoproteins which are not extracted by Triton X-100 also have a more rapid turnover, as indicated by the 80–155% higher specific activity of hexosamine and sialic acid 1 day after labeling with [^{3}H]glucosamine in vivo. The specific activity of sialic acid in the synaptosomal soluble glycoproteins 2 hr after labeling was greater than 100 times that of the synaptosomal particulate fraction, whereas the difference in hexosamine specific activity in these two fractions was only twofold, and by 22 hr there was little or no difference in the specific activities of sialic acid and hexosamine in synaptosomal soluble as compared to membrane glycoproteins. These data indicate that sialic acid may be added locally to synaptosomal soluble glycoproteins before there is significant labeling of nerve ending glycoproteins by axoplasmic transport. Fifty to sixty percent of the hyaluronic acid and heparan sulfate of brain is located in the various membranes comprising the microsomal fraction, whereas half of the chondroitin sulfate is soluble and only one-third is in microsomal membranes. When microsomes are subfractionated on a discontinuous density gradient over half of the hyaluronic acid and chondroitin sulfate are found in membranes with a density less than that of 0.5 *M* sucrose (representing a six- to sevenfold enrichment over their concentrations in the membranes applied to the gradient), whereas half of the heparan sulfate is present in membranes with a density greater than that of 0.8 *M* sucrose. The three glycosaminoglycans found in brain therefore appear to have specific localizations both with respect to the soluble and particulate fractions as well as among a variety of membrane types, most of which probably originate from cell structures other than the endoplasmic reticulum. Appreciable concentrations of glycosaminoglycans were also found in purified axons from myelinated nerves. After labeling the glycosaminoglycans with glucosamine in vivo, it was found that the specific activity of heparan sulfate relative to that of chondroitin sulfate was very similar in both axons and neuronal perikarya. However, the almost tenfold difference in the relative specific activity of hyaluronic acid in these two neuronal subfractions indicates that the axonal hyaluronic acid is either not representative of the entire metabolic pool present in the cell body, or that sulfated and nonsulfated glycosaminoglycans are transported by axoplasmic flow at different rates.

Glycoproteins have been reported as being present in a variety of nervous tissue membranes, including myelin (Quarles et al., 1973; Matthieu et al., 1974; Wood and McLaughlin, 1975), synaptic membranes and synaptic vesicles (Breckenridge and Morgan, 1972; Morgan et al., 1973, Gurd and Mahler, 1974; Zanetta et al., 1975), and surface membranes of neuroblastoma cells (Brown, 1971; Glick et al., 1973; Truding et al., 1974). They are also found in cell bodies of neurons and glia (Margolis and Margolis, 1974; Van Nieuw Amerongen et al., 1974). Recent evidence suggests that these glycoproteins may mediate a number of specific cell–cell interactions, including intercellular adhesion and the mechanisms governing neural histogenesis, regional brain differentiation, and the specificity of neuronal associations (Garber and Moscona, 1972a,b; Hausman and Moscona, 1975). A small proportion of the brain glycoproteins (ca. 10%) occurs in a soluble form, and has a significantly more rapid turnover than the membrane glycoproteins (Margolis and Gomez, 1973; Margolis and Margolis, 1973a,b).

The distribution of glycosaminoglycans (mucopolysaccharides) is less well defined. Over half of the chondroitin sulfate in brain is present as a soluble proteoglycan in which the polysaccharide chains are covalently linked by *O*-glycosidic bonds to serine residues in the protein moiety, while most of the hyaluronic acid and heparan sulfate are particulate (Margolis et al., 1972; Margolis and Margolis, 1973b). However, during early stages of brain development (e.g., in 1 week-old rats) there are large amounts of soluble hyaluronic acid, which may provide an extracellular matrix for neuronal migration and differentiation (Margolis et al., 1975). All three glycosaminoglycans of brain are present in varying amounts in neuronal perikarya, astrocytes, and oligodendroglia (Margolis and Margolis, 1974), and chromaffin granules of adrenal medulla contain significant quantities of chondroitin sulfate and heparan sulfate, which are released, together with catecholamines, upon stimulation of the adrenal gland (Margolis and Margolis, 1973c; Margolis

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et al., 1973). These data therefore suggest that the glycosaminoglycans may have a number of functional roles in nervous tissue, including the binding, storage, and release of neurotransmitter amines.

Our previous studies on the distribution, carbohydrate composition, and metabolism of glycoproteins and glycosaminoglycans in neuronal perikarya and glial cells have now been extended to obtain similar information concerning the remaining portions of the neuron (i.e., axons and nerve endings), as well as various subcellular fractions of rat brain, including the membranes of diverse origin comprising the microsomal fraction, the soluble proteins of the synaptosome, cellular and synaptosomal mitochondria, and purified synaptic membranes.

Experimental Section

Synaptosomes and synaptic plasma membranes were isolated from the cerebra (after removal of brainstem and cerebellum) of 25- to 35-day-old Sprague-Dawley rats essentially according to procedure II of Gurd et al. (1974), with the modification that the washed synaptosomes were lysed by homogenization in 1 m*M* phosphate buffer–0.1 m*M* EDTA (pH 7.5). Four volumes of phosphate/EDTA solution (at room temperature) was then added, and after mixing by gentle stirring, the suspension was allowed to stand for 40 min at 4°C. By this modification the synaptosomal soluble proteins (which were not retained by Gurd et al.) could be recovered for analysis.

The synaptic plasma membranes sedimenting at the 0.6/0.8 *M* sucrose interface were diluted with 3 volumes of distilled water and centrifuged for 30 min at 97000*g*. On the basis of enzymic and morphological criteria Gurd et al. (1974) estimated the purity of their isolated synaptic membranes to be 75–80%, and assays of Na^+K^+-ATPase, succinic dehydrogenase, NADPH cytochrome *c* reductase, acid phosphatase, and monoamine oxidase in our synaptic membrane and other fractions indicated a similar degree of purity. The membrane pellets from 40 cerebra were homogenized in 9 ml of 0.2% (v/v) Triton X-100–1 m*M* NaEDTA (pH 7.5), allowed to stand for 10 min at room temperature, and then centrifuged for 30 min at 100000*g*.

Synaptosomal mitochondria were obtained as a pellet below the 1.3 *M* sucrose layer in the density gradient used for the isolation of synaptic membranes, and mitochondria of nonsynaptosomal origin were obtained as a pellet at the bottom of the Ficoll gradient used for the isolation of synaptosomes. They were further purified by resuspending in 0.32 *M* sucrose and sedimenting through 1.3 *M* sucrose (90 min, 53000*g*).

Microsomes were obtained by homogenizing cerebra in 9 vol of 0.32 *M* sucrose by the same procedure used for the isolation of synaptic membranes, centrifuging for 30 min at 12000*g*, and washing the pellet once by rehomogenizing in the same volume of sucrose solution as used previously. The combined supernatants were then centrifuged for 2 hr at 140000*g*. The 140000*g* supernatant was saved as the soluble fraction, and the microsomal pellets were either used directly for analysis or subfractionated (18 hr, 97000*g*) on a discontinuous sucrose gradient consisting of 2.0, 1.3, 1.0, and 0.8 *M* sucrose (Gurd et al., 1974). In other experiments, a fraction of "light" microsomes was obtained by differential centrifugation of a sucrose homogenate of brain in the same manner as described above, except that the fraction used was that sedimenting between 25000*g* and 140000*g*. This was then subfractionated (18 hr, 97,000*g*) on a discontinuous sucrose gradient consisting of 0.8, 0.7, 0.6, and 0.5 *M* sucrose.

Axons (free of myelin) were prepared from bovine white matter by the procedure of DeVries et al. (1972), and their purity was checked by phase contrast microscopy. In order to study the composition and relative labeling of glycosaminoglycans in axons of rat brain, myelinated axons were prepared from pooled frozen rat brainstems by the same procedure used for the isolation of axons from bovine white matter. However, the floating layer of myelinated axons was rewashed much more extensively (four to five times) until a floating layer free of nuclei, capillaries, and other entrapped contaminants was obtained. Since these repeated washings entailed a significant degree of lysis and a decreased yield of myelinated axons, the myelinated axons were not further lysed by suspension in hypotonic buffer, but were instead used directly for the isolation of glycosaminoglycans, which we have previously shown to be absent from myelin (Margolis, 1967).

To obtain subcellular fractions in which the hexosamine and sialic acid residues of the glycoproteins, glycosaminoglycans, and gangliosides were labeled, 20–80 rats were injected intraperitoneally with [1-^{3}H]glucosamine (Schwarz/Mann, 6.5 Ci/mmol, 3.4–3.8 μCi/g body weight) and decapitated after 2 or 22 hr. The yield of each labeled fraction was noted, and they were then mixed with unlabeled carrier fractions obtained from 60 to 400 brains prior to analysis of their complex carbohydrates. Specific activities of hexosamine and sialic acid in the isolated glycopeptides, glycosaminoglycans, and gangliosides were corrected for dilution to calculate their specific activities in the labeled fractions.

Pooled subcellular fractions were dialyzed, lyophilized, suspended in 0.15 *M* NaCl (1 ml/250 mg of lyophilized dry weight), and extracted with chloroform–methanol (2:1, v/v, followed by chloroform–methanol in the reverse ratio of 1:2). In certain cases the combined chloroform–methanol extracts were saved for measurement of the concentrations and labeling of gangliosides in various subcellular fractions (in preparation). The lipid-free protein residue was dried in vacuo, suspended at a concentration of 2% in boric acid–borax buffer (pH 7.8), and digested with Pronase for a total of 48 hr at 55°C as described previously (Margolis and Margolis, 1970). A small amount of undigested material was removed by centrifugation and the Pronase digest was desalted by gel filtration on Sephadex G-15. The solution was then concentrated to a volume of 5–7 ml/100 mg of original lipid-free dry weight, and made 0.04 *M* in NaCl. Glycosaminoglycans were precipitated with cetylpyridinium chloride (Margolis and Margolis, 1972b), and excess cetylpyridinium chloride was removed from the supernatant solution, containing the glycopeptides derived from the glycoproteins, by extraction with *n*-amyl alcohol.

The sulfated glycosaminoglycans were separated from hyaluronic acid by differential precipitation with cetylpyridinium chloride from 0.3 *M* NaCl, and excess cetylpyridinium chloride was removed from the hyaluronic acid by precipitation with KSCN followed by dialysis. The concentration of hyaluronic acid in the different cell fractions was determined from the glucosamine content of the supernatant obtained after precipitating the sulfated glycosaminoglycans from 0.3 *M* NaCl, and heparan sulfate and chondroitin sulfate concentrations were based on the glucosamine and galactosamine content of the sulfated glycosaminoglycan fraction. Glycosaminoglycans were hydrolyzed for 3 hr at 100°C in 6 *N* HCl, and glucosamine and galactosamine

Table I: Glycoprotein Carbohydrate Composition of Mitochondria, Microsomes, and Whole Rat Brain.

	Mitochondria		Microsomes		Whole Brain	
	(μmol/100 mg of LFDW[a])	Molar Ratio	(μmol/100 mg of LFDW)	Molar Ratio	(μmol/100 mg of LFDW)	Molar Ratio
Mannose	2.160	1.00	3.623	1.00	1.995	1.00
N-Acetylglucosamine	0.565	0.26	4.045	1.12	2.136	1.07
N-Acetylgalactosamine	0.028	0.01	0.334	0.09	0.250	0.13
Galactose	0.273	0.13	1.997	0.55	1.074	0.54
Fucose	0.143	0.07	1.255	0.35	0.745	0.37
N-Acetylneuraminic acid	0.183	0.09	1.756	0.49	0.876	0.44
Percent carbohydrate[b]	0.7		2.7		1.5	

[a] LFDW = lipid-free dry weight. [b] Percent by weight of lipid-free dry weight.

Table II: Glycoprotein Carbohydrate Composition of Subsynaptosomal Fractions of Rat Brain.

					Synaptic Membranes					
	Soluble Protein		Mitochondria		Total Membranes		Triton X-100 Extract		Triton X-100 Residue	
Mannose	1.819[a]	1.00[b]	3.490	1.00	5.364	1.00	7.994	1.00	3.978	1.00
N-Acetylglucosamine	0.558	0.31	1.181	0.34	2.865	0.53	6.403	0.80	1.000	0.25
N-Acetylgalactosamine	0.050	0.03	0.070	0.02	0.139	0.03	0.359	0.05	0.023	0.01
Galactose	0.399	0.22	0.723	0.21	1.502	0.28	3.506	0.44	0.446	0.11
Fucose	0.274	0.15	0.414	0.12	0.845	0.16	1.998	0.25	0.238	0.06
N-Acetylneuraminic acid	0.169	0.09	0.432	0.12	0.987	0.18	2.270	0.28	0.309	0.08
Percent carbohydrate[c]	0.6		1.2		2.3		4.6		1.2	

[a] Concentration, expressed as micromoles per 100 mg of lipid-free dry weight. [b] Molar ratios. [c] Percent by weight of lipid-free dry weight.

were determined using the amino acid analyzer.

Sialic acid in the glycopeptides was determined by the periodate–resorcinol method of Jourdian et al. (1971), and glucosamine and galactosamine were quantitated using the amino acid analyzer after hydrolysis of the glycopeptides for 8 hr at 100°C in 4 *N* HCl. Galactose was determined enzymatically as described previously (Margolis and Margolis, 1970), mannose, fucose, and glucose were measured enzymatically by the procedure of Finch et al. (1969), and fructose was determined by the method of Bernt and Bergmeyer (1974).

To determine the specific activity of hexosamine and sialic acid in the glycoproteins, glycopeptides were desialylated by mild acid hydrolysis (0.1 *N* H_2SO_4, 1 hr, 80°C) and free sialic acid was separated from the desialylated glycopeptides by gel filtration on Sephadex G-15. This procedure yields radiochemically pure fractions of hexosamine-labeled glycopeptides and sialic acid (Margolis and Margolis, 1973a). The specific activities of chondroitin sulfate and heparan sulfate were determined after digestion with chondroitinase ABC using methods described previously (Margolis and Margolis, 1972a, 1973a).

Results and Discussion

Distribution of Glycoproteins in Subcellular Fractions. We have previously reported (Margolis and Margolis, 1973b) that approximately 90% of the brain glycoproteins are present in the particulate fraction, indicating that they are largely membrane components. However, very little information is available concerning the concentration and carbohydrate composition of glycoproteins in subcellular fractions of brain. The glycoprotein carbohydrate composition of mitochondria, microsomes, and various subsynaptosomal fractions of rat brain is given in Tables I and II, and compared with that of whole brain. It was found that the concentration of glycoproteins was highest in the synaptic and microsomal membranes, where their levels were 50–80% higher than in whole rat brain. The glycoproteins of microsomes and whole brain are similar insofar as mannose comprises a relatively smaller proportion of the total carbohydrate (28% on a molar basis) than in the other subcellular fractions studied, where mannose accounts for 46–64% of the total sugar present.

The concentration of glycoproteins in cellular mitochondria (Table I) and in the soluble protein of the synapse is only one-half of that in whole brain, whereas their concentration in synaptic mitochondria (Table II) is almost twice that of cellular mitochondria.

The synaptic membrane fraction was also extracted with Triton X-100 using conditions reported to selectively solubilize the synaptic plasma membrane and leave synaptic junctional complexes intact (Cotman et al., 1971; Davis and Bloom, 1973). This procedure extracted 70% of the synaptic membrane glycoproteins but only 35% of the lipid-free dry weight, and the glycoproteins extracted by and resistant to Triton X-100 differ in both their carbohydrate composition (Table II) and turnover (see below).

The concentration of glycoproteins (1.1% carbohydrate by weight of the lipid-free dry weight) in axons prepared from bovine white matter is identical with that previously reported for neuronal perikarya from rat and bovine brain, although there are considerable differences in the sugar composition of the two neuronal subfractions (Margolis and Margolis, 1974, and Table III). Such marked differences in carbohydrate composition are not surprising insofar as the axonal fraction has been shown to consist largely of neurofilaments (DeVries et al., 1972), whereas the cell body contains many additional components.

Presence of Glucose in Glycopeptides. We have previously reported the presence of small amounts of glucose in gly-

Table III: Glycoprotein Carbohydrate Composition of Axons Prepared from Bovine White Matter.

	μmol/100 mg of Lipid-Free Dry Weight[a]	Molar Ratio
Mannose	2.250	1.00
N-Acetylglucosamine	0.968	0.43
N-Acetylgalactosamine	0.295	0.13
Galactose	1.056	0.47
Fucose	0.238	0.11
N-Acetylneuraminic acid	0.540	0.24

[a] Total carbohydrate = 1.1% by weight of the lipid-free dry weight.

Table IV: Glucose Content of Glycopeptides Prepared from Subcellular Fractions of Brain.

	μmol of Glucose/100 mg of Lipid-free Dry Weight	Percent of Non-glucose Carbohydrate
Whole brain (30 days old)	0.12	3
Microsomes	0.70	5
Mitochondria	6.52	181
Axons (bovine)	6.07	101
Synaptosomal soluble protein	10.20	291
Synaptic membranes	7.42	57
Synaptosomal mitochondria	9.56	139

copeptides prepared from adult rat and rabbit brain (Margolis et al., 1972), and have recently found much larger quantities in glycopeptides from young rat brain. On the basis of its presence in a high molecular weight polymer which can be completely separated from the glycopeptides by gel filtration on Sephadex G-50, and the almost quantitative yield of monomeric glucose from this material after digestion with amyloglucosidase from *Aspergillus niger*, we have identified the glucose found in brain glycopeptide preparations as a metabolically stable form of glycogen, or limit dextrins produced as a result of glycogen degradation (unpublished results). Although brains from rats of the age used in the present subcellular fractionation studies contain very little (i.e., essentially adult levels) of this material, we found quite large amounts of glucose in glycopeptides prepared from all subcellular fractions except for microsomes, and in a number of cases the amount of glucose is two to three times the sum of all of the other sugars present (Table IV). In distinction to our results with glycopeptides prepared from whole brain of very young rats (less than 1 week of age), amyloglucosidase treatment of glycopeptides prepared from subcellular fractions of 30-day-old rat brain released only 8% of the total glucose present (as determined after acid hydrolysis). It appeared that this glucose might originate from sucrose which was firmly bound by glycoprotein "lectins" present in the subcellular fractions, although we considered it unlikely that such binding would persist even after extensive dialysis followed by digestion of the glycoproteins with Pronase. However, to examine this possibility glycopeptides were treated with yeast invertase and also submitted to mild acid hydrolysis (3 *N* HCl, 15 min, 68°C). These procedures released only 6 and 10%, respectively, of the expected amounts of glucose and/or fructose based on the total glucose present in glycopeptides from the various subcellular fractions, although they gave quantitative yields of glucose and/or fructose from sucrose or inulin controls.

Table V: Specific Activities of Hexosamine and Sialic Acid in Subcellular Fractions of Rat Brain 2 and 22 Hr after Administration of [1-^{3}H]Glucosamine.

	Hexosamine[a]	RSA[b]	Sialic Acid[a]	RSA[b]
2 hr				
Synaptosomal soluble	3100	2.21	45700	110
Synaptosomal particulate	1400	1.00	417	1.00
22 hr				
Synaptosomal soluble	109000	1.00	55500	1.32
Synaptic membranes	109000	1.00	42100	1.00
Synaptosomal mitochondria	102300	0.94	36900	0.88
Nonsynaptosomal mitochondria	157300	1.44	67100	1.59

[a] Specific activity as cpm/μmol. [b] RSA = relative specific activity.

Other laboratories have also found significant amounts of glucose in synaptic membrane glycoproteins (Zanetta et al., 1975) and in glycopeptides prepared from rat liver mitochondria (Itoh et al., 1974). In agreement with Zanetta et al. (1975) we find that subcellular fractions prepared without Ficoll (e.g., our microsomal fraction) contain much less glucose than those in which Ficoll was a component of the isolation medium. However, contamination with Ficoll present in the density gradients does not appear to be a sufficient explanation, since axons also contained relatively large amounts of "glycoprotein" glucose (Table IV) even though Ficoll was not used in their preparation. Therefore, the origin of the glucose found in most of our subcellular fractions remains unknown.

Metabolism of Glycoproteins in Subcellular Fractions. We have previously reported that the turnover half-times for hexosamine and sialic acid in various pools of brain glycoproteins range from approximately 1–4 weeks (Margolis and Margolis, 1973a). Based on this information, it can be concluded that specific activities of glycoproteins measured in subcellular fractions within 1 day after labeling should be roughly proportional to the turnover rates of these glycoproteins. At 2 hr after administration of labeled glucosamine the specific activity of sialic acid in the soluble glycoproteins at the synapse was greater than 100-fold that of the particulate glycoproteins, while the specific activity of hexosamine was only twice that found in the particulate fraction (Table V). By 22 hr after the administration of labeled precursor the specific activities of hexosamine and sialic acid in synaptosomal soluble and particulate glycoproteins are approximately equal[1] (Table V). The very high specific activity of sialic acid relative to that of hexosamine in the soluble synaptic glycoproteins at 2 hr is noteworthy, insofar as the specific activity of sialic acid is usually considerably less than that of hexosamine in both whole brain and in all other subcellular fractions studied at a variety of time points (Margolis and Margolis, 1973a; and Table V), whereas in this case the specific activity of sialic acid is 15 times that of hexosamine. This relationship between the specific activities of sialic acid and hexosamine in soluble and particulate synaptosomal glycoproteins was also found

[1] The specific activity of purified synaptic membranes at 22 hr was within 10% of that of the total synaptosomal particulate glycoproteins. Therefore, the values given in Table V for synaptic membranes can be considered representative of the entire particulate fraction for purposes of comparison with specific activities determined at 2 hr.

Table VI: Distribution of Glycosaminoglycans in Subcellular Fractions Obtained by Differential Centrifugation of Rat Brain.[a]

	Hyaluronic Acid		Heparan Sulfate		Chondroitin Sulfate	
	Concn[b]	Percent[c]	Concn	Percent	Concn	Percent
Nuclei and cell debris	0.142	19	0.074	21	0.209	12
Crude mitochondrial fraction	0.112	5	0.035	3	0.064	1
"Heavy" microsomes	0.126	28	0.071	34	0.190	19
"Light" microsomes	0.223	26	0.102	25	0.344	17
Soluble fraction	0.169	22	0.057	17	0.860	51

[a] Subcellular fractions were prepared as follows: A 10% homogenate of 30-day-old rat brain in 0.32 *M* sucrose was centrifuged for 8 min at 1000*g* and the pellet washed once to obtain the *nuclei and cell debris*; combined supernatants from above were centrifuged for 20 min at 10000*g* and the pellet washed once to yield a crude *mitochondrial fraction*; supernatants from the crude mitochondria were centrifuged for 30 min at 25,000*g* to yield the *"heavy" microsomes*; and the supernatant from the heavy microsome fraction was centrifuged for 2 hr at 140000*g* to obtain the *"light" microsomes* and the *soluble fraction.* [b] Concentration expressed as micromoles of constituent hexosamine per 100 mg of lipid-free dry weight. [c] Represents the percent of the total in whole brain which is found in each fraction (sum of figures in each column = 100%). Whole brain concentrations (micromoles of glucosamine or galactosamine per gram wet weight of brain) of hyaluronic acid, heparan sulfate, and chondroitin sulfate are 0.102, 0.062, and 0.302, respectively.

to be highly reproducible, insofar as essentially identical results were obtained in two separate experiments.[2]

Although our data clearly demonstrate the rapid turnover of sialic acid in the soluble glycoproteins of the synapse, and indicate that this sialic acid has been added locally, there is still considerable controversy concerning the possible presence of glycosyltransferases at nerve endings (Raghupathy et al., 1972; Barondes, 1974; Den et al., 1975). It is known that after a somewhat longer interval (3 hr) there is relatively little labeling of nerve ending proteins by leucine, even though the incorporation of glucosamine is extensive in this time period (Barondes, 1968). These data have been interpreted as evidence for the local addition of sugars at nerve endings at a time too early for the arrival by axoplasmic flow of glycoproteins synthesized in the cell body by the usual mechanisms involving glycosyltransferases in the endoplasmic reticulum and Golgi apparatus (Barondes, 1968, 1974). However, the possibility remained that hexosamine and sialic acid might still be added in the endoplasmic reticulum to already synthesized acceptor proteins (hence the low labeling with leucine), rather than after transport of such acceptors to the nerve ending. Our observation that by 2 hr sialic acid attains an uncharacteristically high specific activity in one nerve-ending subfraction lends support to the hypothesis that this sugar, at least, may be added locally to the synaptic soluble proteins, since the much lower labeling of hexosamine provides a measure of the maximum labeling which might be expected in this short time period due to the arrival of labeled glycoproteins by axoplasmic flow. It is not known whether these rapidly labeled soluble glycoproteins may have a particular role in synaptic transmission or other nerve ending functions. However, their specific activities at 2 and 22 hr in comparison with those of synaptic membranes are consistent with (but in no way conclusive evidence of) the soluble glycoproteins being metabolic precursors of synaptic membranes.

Although the glycoprotein concentration of the synaptic mitochondria was only half that of the synaptic membranes, their carbohydrate compositions were similar, with the exception that the mitochondria had a considerably higher proportion of mannose as compared to the other sugars (Table II). The specific activities of hexosamine and sialic acid in the synaptic membrane and mitochondrial fractions were also quite similar. However, the synaptosomal mitochondria had a considerably lower specific activity than the cellular mitochondria (Table V), as well as a higher concentration and a somewhat different composition of glycoprotein carbohydrate (Tables I and II).

As was pointed out above, the concentration of glycoproteins in the Triton X-100 extract of synaptic membranes is almost four times that of the material resistant to Triton extraction, and the glycoproteins of these two synaptic membrane subfractions also differ in their carbohydrate composition (Table II). Labeling experiments demonstrated additional differences in the metabolic activity of these two fractions, insofar as the specific activities of hexosamine and sialic acid in the Triton extract of synaptic membranes (after 22 hr of labeling) were 91600 and 31700 cpm/μmol, respectively, while the corresponding specific activities in the Triton-unextractable residue were 163500 and 80800 cpm/μmol. Thus, the specific activities were 80–155% greater in the synaptic membrane glycoproteins which were resistant to extraction with Triton X-100.

Distribution and Metabolism of Glycosaminoglycans. To obtain information concerning the distribution of glycosaminoglycans among the major subcellular fractions of brain, a homogenate of 30-day-old rat brain was fractionated by differential centrifugation (Table VI). The fraction sedimenting at 1000*g* contained nuclei, unbroken cells, blood vessels, and cell debris; the crude mitochondrial fraction contained myelin and broken-off nerve endings ("synaptosomes") in addition to mitochondria; and the microsomal fractions consisted of plasma membranes of neurons and glial cells as well as internal membranes from the endoplasmic reticulum.

Approximately 50–60% of the hyaluronic acid and heparan sulfate was found in the microsomal fraction, while, as noted previously (Margolis and Margolis, 1973b), half of the chondroitin sulfate occurs in a soluble form (Table VI). The crude mitochondrial fraction had a low concentration of glycosaminoglycans, containing only 1–5% of the total amount present in brain. However, because of the heterogeneity of this fraction and the functional importance of the nerve endings which are also present here, glycosaminoglycans were analyzed in a number of purified subfractions obtained from the crude "mitochondria". These subfractions

[2] The soluble glycoproteins account for 14 and 29% of the synaptosomal glycoprotein sialic acid and hexosamine, respectively, and for 40% of the radioactivity incorporated into synaptic glycoproteins by 2 hr after the administration of labeled glucosamine. After this period of labeling sialic acid contains 81% of the total radioactivity in the synaptosomal soluble glycoproteins, as compared to the usual 10–12% found in other subcellular fractions, or in the synaptic soluble glycoproteins after 22 hr.

Table VII: Concentration of Glycosaminoglycans in Mitochondria and Synaptosomal Subfractions of Rat Brain.[a]

	Hyaluronic Acid	Heparan Sulfate	Chondroitin Sulfate
Mitochondria	0.013	0.018	0.018
Synaptosomal soluble protein	0.036	0.016	0.026
Synaptosomal particulate protein	0.097	0.038	0.054
Synaptosomal mitochondria	0.053	0.026	0.031
Synaptic membranes	0.024	0.029	0.038
0.2% Triton X-100 extract	0.032	0.063	0.067
Triton-unextractable residue	0.019	0.011	0.023

[a] Expressed as micromoles of constituent hexosamine per 100 mg of lipid-free dry weight.

Table VIII: Distribution of Glycosaminoglycans in Rat Brain Microsomal Subfractions.[a]

	Hyaluronic Acid		Heparan Sulfate		Chondroitin Sulfate	
	Concn[b]	Percent	Concn	Percent	Concn	Percent
0.8 *M* sucrose	0.608	76	0.160	45	0.857	70
1.0 *M* sucrose	0.061	9	0.066	22	0.135	13
1.3 *M* sucrose	0.054	9	0.054	20	0.086	9
2.0 *M* sucrose	0.084	6	0.080	13	0.150	8
Unfractionated	0.197	100	0.098	100	0.322	100

[a] Microsomes were obtained by centrifuging a 10% homogenate of 30-day-old rat cerebrum (in 0.32 *M* sucrose) for 30 min at 12,000*g*, washing the pellet once, and centrifuging the combined supernatants for 2 hr at 140000*g*. A portion was then subfractionated on a discontinuous sucrose gradient (18 hr, 97,000*g*). [b] Expressed as micromoles of constituent hexosamine per 100 mg of lipid-free dry weight.

had relatively low concentrations of glycosaminoglycans, although the synaptosomes had an appreciable content of hyaluronic acid, most of which was particulate (Table VII). Preliminary studies indicate that much of the glycosaminoglycans in the synaptosomal particulate fraction may be located in synaptic vesicles, and that such vesicles isolated from brain differ from chromaffin granules of adrenal medulla, which contain only sulfated glycosaminoglycans (Margolis and Margolis, 1973c; Margolis et al., 1973). These possibilities are now being investigated further in the context of a more systematic study of the complex carbohydrate composition of synaptic vesicles obtained from central nervous tissue.

As is the case for the glycoproteins, most of the glycosaminoglycans in the purified synaptic membranes are extracted by Triton X-100. Their concentration in the cellular mitochondria was very low, although it was somewhat higher in mitochondria of synaptosomal origin. However, it is not presently clear whether these low levels of glycosaminoglycans in mitochondria and synaptic membranes represent real constituents or merely reflect a small degree of contamination by microsomal or other membranes. Because of the small amounts of glycosaminoglycans and the relatively low yields of purified synaptosomal subfractions, the labeled material obtained after administration of [^{3}H]glucosamine was insufficient to permit meaningful conclusions concerning the metabolism of glycosaminoglycans at nerve endings.

Subfractionation on a discontinuous sucrose gradient of the membranes sedimenting between 12000 and 140000*g* revealed that 70–80% of the hyaluronic acid and chondroitin sulfate was found in membranes having a relatively low density (i.e., floating on 0.8 *M* sucrose), while only 45% of the microsomal heparan sulfate was in this fraction (Table VIII). When a preparation of "light" microsomes was fractionated on a discontinuous density gradient ranging from 0.5 to 0.8 *M* sucrose, 50–60% of the hyaluronic acid and chondroitin sulfate was found in membranes with a density less than that of 0.5 *M* sucrose (Table IX), representing a six- to sevenfold enrichment over the concentration of these two glycosaminoglycans present in the material applied to the gradient, and a 14-fold enrichment over the concentration of hyaluronic acid in whole brain. In distinction to the distribution of hyaluronic acid and chondroitin sulfate, half of the heparan sulfate was present in membranes with a density greater than that of 0.8 *M* sucrose, and only 17% was found on the 0.5 *M* sucrose layer (Tables VIII and IX). It is therefore evident that the three glycosaminoglycans differ significantly in their distribution in brain. Approximately half of the chondroitin sulfate is present in the form of soluble proteoglycans, and the remainder occurs in membranes of very low density together with most of the hyaluronic acid. Over 80% of the heparan sulfate is also particulate, but in this case there is a shift in its distribution (as compared to hyaluronic acid and chondroitin sulfate) to membranes of somewhat greater density. Preliminary studies of the microsomal subfractions (and their comparison with similar fractions prepared from liver) indicate that the low density membranes, having the highest concentration of glycosaminoglycans, are probably derived in large part from neuronal and glial plasma membranes rather than from endoplasmic reticulum. The turnover half-times of the different metabolic pools of brain glycosaminoglycans, which range from 4 to 45 days (Margolis and Margolis, 1973a), are also considerably less rapid than those reported for endoplasmic reticulum. A more detailed investigation of the enzymic and chemical composition, metabolism, and morphology of these membrane subfractions rich in glycosaminoglycans is currently in progress.

Table IX: Distribution of Glycosaminoglycans in Subfractions of "Light" Microsomes from Rat Brain.[a]

	Hyaluronic Acid		Heparan Sulfate		Chondroitin Sulfate	
	Concn[b]	Percent	Concn	Percent	Concn	Percent
0.5 *M* sucrose	1.457	59	0.208	17	1.992	49
0.6 *M* sucrose	0.206	13	0.128	17	0.631	24
0.7 *M* sucrose	0.089	4	0.084	8	0.187	5
0.8 *M* sucrose	0.103	5	0.104	10	0.126	4
Pellet	0.067	19	0.080	48	0.106	18
Unfractionated	0.208	100	0.100	100	0.344	100

[a] Microsomes were prepared as in Table VIII except that the membranes sedimenting between 25,000*g* and 140,000*g* were used for subfractionation. [b] Expressed as micromoles of constituent hexosamine per 100 mg of lipid-free dry weight.

Although very little glycosaminoglycan was found in isolated nerve endings prepared by the methods used in this study, appreciable amounts are present in axons from myelinated nerves (Table X). The concentration in bovine axons is approximately 60% of that previously reported for bovine neuronal perikarya (Margolis and Margolis, 1974), and there are significant differences in the relative amounts of hyaluronic acid and chondroitin sulfate in these two neuronal subfractions. Axons were also isolated from rat brainstem in order to study the labeling of the glycosaminogly-

Table X: Distribution of Glycosaminoglycans in Axons and Neuronal Perikarya.

	Rat Brain		Bovine Brain	
	Axons[a]	Neuronal Perikarya[b]	Axons	Neuronal Perikarya[b]
Hyaluronic acid	0.076[c] (28)[d]	0.108 (17)	0.221 (43)	0.159 (19)
Heparan sulfate	0.026 (10)	0.122 (19)	0.081 (16)	0.137 (17)
Chondroitin sulfate	0.168 (62)	0.414 (63)	0.206 (41)	0.534 (64)
Total	0.270	0.644	0.508	0.830

[a] Includes myelin protein. [b] From Margolis and Margolis, 1974. [c] Concentration, expressed as micromoles of constituent hexosamine per 100 mg of lipid-free dry weight. [d] Percentage of total glycosaminoglycan.

Table XI: Relative Specific Activities of Glycosaminoglycans in Neuronal Perikarya and Axons of Rat Brain.

	Axons[a]		Neuronal Perikarya[b]
	Specific Activity	RSA	RSA
Chondroitin sulfate	80700	1.0	1.0
Heparan sulfate	242500	3.0	3.3
Hyaluronic acid	27400	0.3	2.6

[a] Isolated 22 hr after administration of [1-^{3}H]glucosamine. Specific activities are expressed as cpm/μmol of constituent hexosamine; RSA = relative specific activity. [b] Isolated 16 hr after administration of [1-^{3}H]glucosamine. Relative specific activities calculated from data in Margolis and Margolis (1974).

cans. Since in this case it was necessary to analyze the entire axon including the considerable amount of myelin protein which is devoid of glycosaminoglycans (see Experimental Section), the concentration given in Table X is lower than would be found for axons after removal of myelin. In both axons and neuronal perikarya from rat brain chondroitin sulfate comprises 62–63% of the total glycosaminoglycan, but there are significant differences in the amounts of hyaluronic acid and heparan sulfate (Table X), as well as in the relative specific activity of hyaluronic acid in the axon as compared to the nerve cell body (Table XI). These relative specific activities were determined in axons 22 hr after the administration of labeled glucosamine, and are compared with neuronal perikarya labeled for a somewhat shorter time period (16 hr). It is not possible to estimate exactly what time differential should be chosen so as to allow an accurate comparison between recently synthesized glycosaminoglycans still present in the nerve cell body and those transported to the axon by axoplasmic flow. However, it is apparent from the almost tenfold difference between the relative specific activity of hyaluronic acid in axons and neuronal perikarya (Table XI) that the axonal hyaluronic acid is either not representative of the entire metabolic pool present in the cell body, or that the sulfated and nonsulfated glycosaminoglycans are transported by axoplasmic flow at different rates. Based on our knowledge that relatively high concentrations of glycosaminoglycans are found in both nerve cell bodies and axons, and that they are transported down the axon by axoplasmic flow (Elam et al., 1970; Elam and Agranoff, 1971), their presence in only very low amounts in isolated nerve endings indicates that they are rapidly metabolized there or otherwise removed, or are possibly present in nerve-ending structures which are not isolated by conventional techniques used for the preparation of synaptosomes.

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An Affinity Adsorbent Containing Deoxyguanosine 5′-Triphosphate Linked to Sepharose and Its Use for Large Scale Preparation of Ribonucleotide Reductase of *Lactobacillus leichmannii*†

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ABSTRACT: P^3-(6-(*N*-Trifluoroacetyl)aminohex-1-yl) deoxyguanosine triphosphate has been prepared by the reaction of *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate with the imidazolide of dGMP and has been characterized. This compound and the corresponding free amine, obtained by removal of the protective trifluoroacetyl group, are activators of ribonucleotide reductase of *Lactobacillus leichmannii.* An affinity adsorbent for the reductase, prepared by reaction of the amine derivative with CNBr-activated Sepharose, contains dGTP covalently attached through the γ-phosphate via a six-carbon chain to the matrix. The method of synthesis of the dGTP derivative is generally applicable to the synthesis of P^3-(ω-aminoalk-1-yl)nucleoside triphosphate esters for the preparation of analogous affinity adsorbents. Ribonucleotide reductase can be rapidly purified to homogeneity, on a large scale, by use of dGTP–Sepharose and conditions for optimum recovery of the enzyme have been determined. The affinity of ribonucleotide reductase and other proteins for dGTP–Sepharose is increased by either raising the ionic strength or lowering the temperature of the eluent. Elution of the enzyme from the adsorbent can be achieved between pH 5.8 and 7.3, whereas at pH 5.3 the reductase is bound extremely tightly and cannot be recovered. Ribonucleotide reductase can be eluted from the adsorbent with dGTP or urea. Elution with urea is carried out at pH 6.3, where the enzyme is stable and maximum recovery is obtained. Affinity chromatography consistently produces ribonucleotide reductase of high specific activity (170–180 units/mg). In the presence of 0.1 to 1.2 *M* urea or hydroxyurea, the enzyme is inhibited, but allosteric activation is unchanged. No alteration in the structure or function of the reductase was detected when the enzyme was exposed to 2.0 *M* urea during elution from the affinity adsorbent, but exposure for longer periods causes some inactivation.

The allosteric ribonucleotide reductase of *Lactobacillus leichmannii* has been shown to be a monomeric enzyme with a single polypeptide chain (Panagou et al., 1972). The allosteric effectors, which are the deoxyribonucleotide products of the reaction catalyzed by the enzyme, show a pattern of specific activation. For example, dGTP specifically activates ATP reduction and dATP specifically activates CTP reduction (Vitols et al., 1967).

Although the enzyme has previously been purified to homogeneity by conventional procedures (Panagou et al., 1972), different preparations were not of the same high specific activity. Instead, the final purification step, preparative polyacrylamide gel electrophoresis, raised the specific activity of all preparations 20–30% irrespective of the specific activity of the sample electrophoresed (Orr et al., 1972). Furthermore, preparative gel electrophoresis is tedious and unsuitable for large scale purification.

It was considered that an affinity adsorbent of one of the

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