Glycosaminoglycans and Glycoproteins Associated with Microsomal Subfractions of Brain and Liver†

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ABSTRACT: Approximately half of the glycosaminoglycans are initially present in the total microsomal fraction after differential centrifugation of a brain homogenate. Most of the hyaluronic acid and chondroitin sulfate is closely associated with a low density subfraction of smooth membranes and can be removed by resedimentation. The easily releasable glycosaminoglycan accounts for 60–65% of the total microsomal hyaluronic acid and chondroitin sulfate, but only 25% of the heparan sulfate, which is firmly bound to microsomal membranes of greater density and appears to have a different subcellular localization than the other glycosaminoglycans. In contrast to the results obtained with brain, the concentration of hyaluronic acid and chondroitin sulfate is very low in rat liver microsomes and these glycosaminoglycans are associated with membrane subfractions of considerably greater density. The concentration of heparan sulfate in liver microsomes is also relatively low (50% of that found in brain), but the two liver microsomal subfractions containing most of this glycosaminoglycan have concentrations somewhat higher than the corresponding subfractions from brain. In metabolic studies, maximum specific activities of hyaluronic acid, heparan sulfate, and glycoprotein hexosamine were observed in the rough endoplasmic reticulum by 2 h after administration of [^{3}H]-glucosamine. At this time maximally labeled chondroitin sulfate is present in the next lower density subfraction, where high specific activity heparan sulfate, hyaluronic acid, and glycoproteins are also found after 18 h. There is then a progressive movement of these labeled complex carbohydrates to still lower density subfractions consisting predominantly of plasma membranes, smooth endoplasmic reticulum, and golgi complex. At 8 days after labeling of the glycosaminoglycans, hyaluronic acid attains a relatively high specific activity in a microsomal subfraction sedimenting at the density of 0.9 M sucrose, suggesting that this population of membranes contains the hyaluronate pool known to have a slow turnover ($t_{1/2}$ = 45 days).

In a previous study of the distribution and metabolism of glycosaminoglycans and glycoproteins in subcellular fractions of brain (Margolis et al., 1975), it was found that half of the total chondroitin sulfate and approximately 20% of the heparan sulfate and hyaluronic acid could be obtained in a soluble form after high speed centrifugation of a brain homogenate. Only very small amounts of these glycosaminoglycans are present in purified mitochondria, myelin, nuclei, or nerve endings (synaptosomes). However, most of the particulate glycosaminoglycans appeared to be associated with low density membranes from the microsomal fraction, which is composed mainly of endoplasmic reticulum, plasma membranes, and golgi apparatus.

To obtain more detailed information on the localization and possible functions of this pool of particulate glycosaminoglycans, we have investigated the distribution and biosynthesis of complex carbohydrates in a number of microsomal subfractions which have also been characterized with respect to their morphology and enzyme composition. The following paper (Krusius et al., 1978) describes certain structural features of glycoproteins in these microsomal and other subcellular fractions of brain, while corresponding data concerning the distribution and metabolism of gangliosides will appear elsewhere.

Methods

Preparation of Microsomal Subfractions. Cerebra and livers from 30- to 40-day-old Sprague-Dawley rats were minced with scissors and homogenized in a glass tissue grinder with 9 volumes of 0.3 M sucrose containing 1 mM NaH_2PO_4/0.1 mM EDTA, pH 7.5, using ten up-down strokes of a loose-fitting Teflon pestle (clearance approximately 0.2 mm). The homogenate was centrifuged for 30 min at 12 000*g* and the supernatant saved. The pellets were resuspended in the original homogenate volume and again centrifuged as above. The combined supernatants were then centrifuged for 2 h at 140 000*g* to obtain the microsomes and a soluble fraction.

Microsomes were resuspended in 0.3 M sucrose (1.6 mL/g of starting tissue) with a few strokes of the homogenizer, and 2.5-mL portions of the suspension were layered onto a gradient consisting of 9 mL each of 1.3, 1.0, 0.8, and 0.5 M sucrose. The pH of all sucrose solutions was checked and adjusted to 7.5 if necessary. In the case of liver microsomes the 0.5 M gradient layer was omitted and a 2 M sucrose layer added. Gradients were centrifuged overnight (approximately 16 h) at 97 000*g* in a swinging bucket rotor (Spinco SW-27), and the fractions concentrating at each interface were aspirated and pooled. The pellet below the 1.3 M sucrose layer (subfraction 5) was also collected and resuspended in 0.3 M sucrose with a few strokes of the homogenizer. A distinct layer of membranes was observed at each sucrose density, and the clearer gradient solution halfway above and below each fraction was aspirated and pooled together with the dense membrane band itself. The average sucrose molarities (after diffusion) at which the different membrane subfractions banded were measured as 0.5, 0.7, 0.9, and 1.1 M for subfractions 1, 2, 3, and 4, respectively.

Gradient layers were either diluted to 0.25–0.3 M sucrose and centrifuged for 1 to 2 h at 140 000*g* to concentrate and

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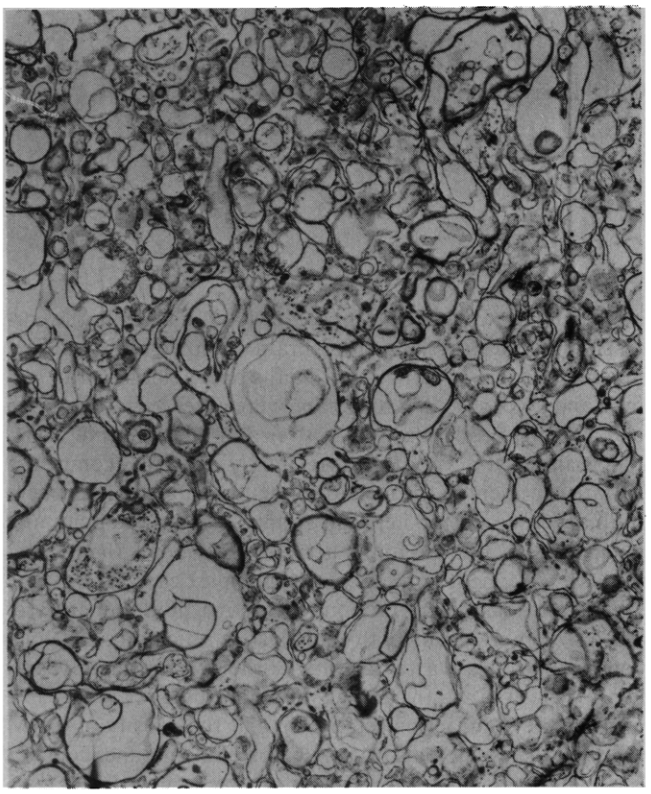

FIGURE 1: Electron micrograph of microsomal subfraction 1 from brain. Magnification 12 000×.

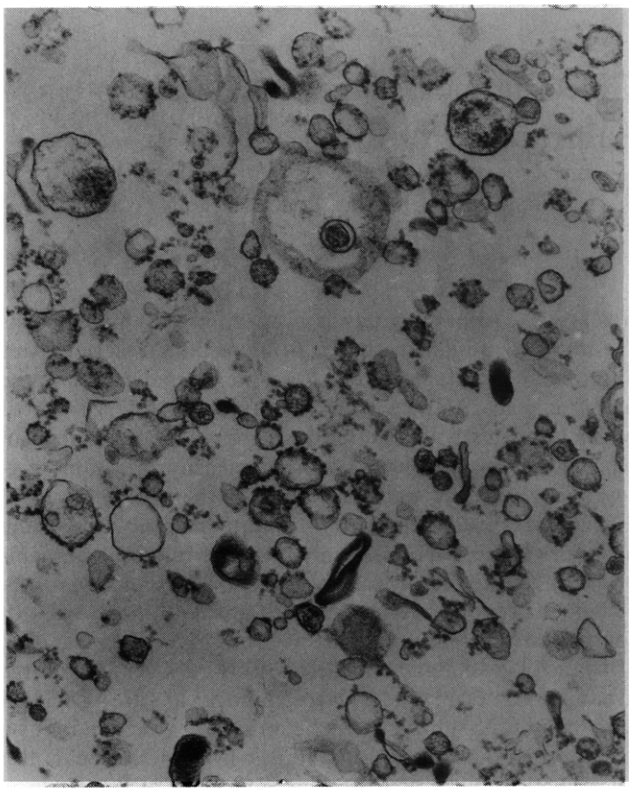

FIGURE 2: Electron micrograph of microsomal subfraction 5 from brain. Magnification 28 000×.

wash the membrane fractions prior to RNA determination and enzyme assays, or the gradient fractions were dialyzed and lyophilized for subsequent analysis of complex carbohydrate composition and specific activity. For these studies glycosaminoglycans and glycoproteins were fractionated and quantified as described previously (Margolis et al., 1975).

Enzyme Assays. $[Na^+ + K^+]$-activated-ouabain-sensitive ATPase (EC 3.6.1.3), 5′-nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9), and phosphodiesterase (EC 3.1.4.1) were assayed under the conditions recommended by Solyom & Trams (1972). Rotenone-insensitive NADPH-cytochrome *c* reductase (EC 1.6.99.1) was assayed by the method of Sottocasa et al. (1967), and acetylcholinesterase was assayed by the method of Ellman et al. (1961). *p*-Nitrophenyl phosphate acid phosphatase (EC 3.1.3.2) was determined as described by Cotman & Matthews (1971), and Mg^{2+}-, K^+-dependent *p*-nitrophenylphosphatase was determined according to Nagai et al. (1966). All enzyme assays were performed under conditions for which the rates were linear with enzyme concentration.

Radioactive Labeling of Microsomal Subfractions. To obtain microsomal subfractions in which the hexosamine and sialic acid residues of the glycosaminoglycans, glycoproteins, and gangliosides were labeled, groups of 9 to 18 rats were injected intraperitoneally with [6-^{3}H]glucosamine (4 to 6 μCi/g of body weight) and brain microsomes were prepared after various time intervals. The yield of protein in each labeled subfraction was noted, and they were then mixed with unlabeled carrier fractions obtained from 35 to 55 brains prior to analysis. Specific activities of glycosaminoglycans, glycoproteins, and gangliosides in the labeled preparations were calculated after correction for dilution with unlabeled membranes.

Electron Microscopy. Microsomal subfractions, suspended in the medium to which they had last been exposed or in 0.3 M sucrose, were fixed by the addition of an equal volume of 4% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4. The suspensions were mixed and allowed to stand for 1 to 2 h at 4 °C. Membranes were collected by centrifugation, using a sample volume calculated to give pellets about 0.5 to 1 mm thick. Samples were "post-fixed" with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4), dehydrated in ethanol, and embedded in Epon, and thin sections were stained with uranyl acetate and lead citrate. They were examined in a Phillips Model 301 electron microscope.

Other Methods. Protein was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard. RNA was determined as described by Fleck & Begg (1965), and in enzyme assays phosphorus was determined by the method of Lazarus & Chou (1972). Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate as described by Weber & Osborn (1969).

Reagents. RNase-free sucrose (Schwarz/Mann) was used throughout. D-[6-^{3}H]Glucosamine (7 Ci/mmol) was purchased from New England Nuclear.

Results

Characterization of Microsomal Subfractions. Electron microscopic examination of the microsomal subfractions from brain revealed that the subfraction of lowest density (no. 1) consisted almost exclusively of large vesicles composed of smooth membranes, with occasional myelin fragments and amorphous material (Figure 1). Subfraction 2 was composed in large part of smooth membranes and of unstacked golgi cisternae, accompanied by mitochondria and nerve endings in various stages of preservation. Subfractions 3 and 4 appeared generally similar to fraction 2, with the distinction that fraction 3 was considerably enriched in golgi cisternae. These two

TABLE I: Composition of Brain Microsomal Subfractions.[a]

	subfraction no.					total microsomes
	1	2	3	4	5	
sucrose molarity	0.5	0.7	0.9	1.1	pellet[b]	
protein (%)	16[c]	19	28	30	7	100
RNA (μg of P/mg of protein)	0.5	0.9	1.2	2.8	10.9	4.7
$[Na^+-K^+]$ATPase	4.4	6.5	5.3	7.1	3.3	5.8
NADPH-cytochrome *c* reductase	0.65	0.36	0.27	0.31	0.39	0.33
phosphodiesterase	0.41	0.50	0.41	0.28	0.14	0.30
K^+-dependent phosphatase	1.9	3.2	3.0	2.5	0.86	2.2
acid phosphatase	1.2	2.0	3.0	3.9	2.3	2.9
acetylcholinesterase	3.4	5.7	3.4	2.8	2.2	9.4

[a] Mean values from three to five determinations. Enzyme activities are expressed as μmol of substrate utilized or product formed per mg of protein per h. [b] Sedimented through 1.3 M sucrose. [c] Only 35% of the protein in this subfraction resediments with the membranes used for RNA and enzyme assays.

subfractions also contained progressively fewer smooth membranes and more fragments of mitochondria, nerve endings, and lysosomes. Subfraction 5 was highly enriched in rough endoplasmic reticulum, with some mitochondrial and lysosomal contamination (Figure 2).

The distribution of protein, RNA, and a number of enzymes known to be associated with plasma membranes in a variety of tissues (Solyom & Trams, 1972) was studied in the total microsomes and in the subfractions derived from them. Rotenone-insensitive NADPH-cytochrome *c* reductase was also assayed as a marker for endoplasmic reticulum. The results of these studies are summarized in Table I.

Subfraction 5 is quantitatively the smallest, containing only 7% of the total microsomal protein. Its high concentration of RNA in comparison with the other subfractions is consistent with its electron microscopic appearance, which indicated that this fraction is largely composed of rough endoplasmic reticulum. Subfractions 2, 3, and 4 together account for 77% of the total microsomal protein. Subfraction 4 has the highest activity of $[Na^+ + K^+]$ATPase, although none of the subfractions are greatly enriched in this plasma membrane marker enzyme. It also has higher concentrations of acid phosphatase activity and of RNA than the lower density subfractions, probably due to the presence of lysosomes and some rough endoplasmic reticulum. Subfractions 2 and 3 are very similar to one another in most respects, although fraction 2 has the highest concentration of acetylcholinesterase. The lowest density subfraction 1, which was found to be composed almost exclusively of smooth membranes, represents only 16% of the total microsomal protein. It has the highest activity of NADPH-cytochrome *c* reductase and low concentrations of RNA and acid and K^+-dependent phosphatases. In addition to the enzymes listed in Table I, most of the subfractions had measurable activities of 5′-nucleotidase and glucose-6-phosphatase, which were, however, too low to draw significant conclusions concerning possible differences between the five subfractions.

The recovery of RNA from the microsomal subfractions averaged 45% of that present in the unfractionated microsomes, probably due to the removal of free ribosomes during resedimentation of the membranes. The recovery of the enzymes listed in Table I averaged 98% (range of 90 to 110%) with the exception of acetylcholinesterase, whose recovery in the subfractions averaged only 34% in several experiments. The reason for the considerable loss in acetylcholinesterase activity during the subfractionation procedure is not known.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that subfractions 2, 3, and 4 have essentially identical polypeptide compositions, while several of the bands present in these three subfractions were missing or considerably more faint in subfraction 5 and in the resedimented membranes of subfraction 1. The electrophoretic pattern of the soluble proteins obtained after resedimenting the subfraction 1 membranes showed many fewer components than were present in the soluble proteins obtained from whole brain. Some bands were common to both soluble protein fractions, and polypeptides of similar size were also seen in the membranes.

On the basis of these data it would appear that subfraction 5 is clearly distinct from the others and is largely composed of rough endoplasmic reticulum, whereas fractions 1 through 4 contain varying proportions of membranes derived from smooth endoplasmic reticulum, plasma membranes, and golgi apparatus. Although fraction 1 membranes are closely associated with very high concentrations of hyaluronic acid and chondroitin sulfate, their enzyme composition and morphological appearance do not allow us to conclude that they are derived from one specific location in the cell.

Distribution of Glycosaminoglycans and Glycoproteins. The distribution of glycosaminoglycans and glycoproteins among the various subfractions is summarized in Table II. It can be seen that, while the concentration of all three glycosaminoglycans increases progressively from membranes of greater to those of lower density, it is primarily hyaluronic acid and chondroitin sulfate which are highly concentrated in subfraction 1. On the other hand, most of the glycoproteins (expressed in terms of hexosamine or sialic acid) are associated with the three subfractions of intermediate density.

For comparison, liver microsomes were subfractionated by a similar procedure, and the relative distribution of glycosaminoglycans in these membranes is summarized in Table III. Because no significant proportion of very low density membranes was obtainable from liver, the lowest concentration of sucrose used in these gradients was 0.8 M, and rough endoplasmic reticulum was sedimented onto a 2 M sucrose cushion rather than being collected as a pellet. It can be seen from Table III that the concentration of chondroitin sulfate and hyaluronic acid in rat liver microsomes is only 5 to 10% of that in brain microsomes, while the concentration of heparan sulfate is half as great in liver as compared with brain. However, in certain higher density membrane subfractions from liver (sedimenting on 1.0 and 1.3 M sucrose) the concentration of heparan sulfate was found to be somewhat greater than in comparable subfractions obtained from brain, whereas the concentration of hyaluronic acid and chondroitin sulfate was lower in all of the liver membrane subfractions. These data suggest that heparan sulfate may share some common function

TABLE II: Concentration of Glycosaminoglycans and Glycoproteins in Microsomal Subfractions of Brain.[a]

subfraction no.	hyaluronic acid	chondroitin sulfate	heparan sulfate	glycoprotein hexosamine	glycoprotein sialic acid
1	0.945	1.581	0.194	2.56	1.16
2	0.189	0.230	0.127	3.20	1.37
3	0.146	0.133	0.091	3.82	1.64
4	0.071	0.091	0.066	3.26	1.27
5	0.044	0.051	0.039	1.67	0.55

[a] Concentration expressed as micromoles per 100 mg of lipid-free dry weight.

TABLE III: Distribution of Glycosaminoglycans in Rat Brain and Liver Microsomal Subfractions.[a]

	hyaluronic acid			chondroitin sulfate			heparan sulfate		
	% of total		concn ratio	% of total		concn ratio	% of total		concn ratio
	brain	liver	(L/B)	brain	liver	(L/B)	brain	liver	(L/B)
0.8 M sucrose[b]	76	6	0.02	70	14	0.02	45	14	0.38
1.0 M sucrose	9	24	0.51	13	19	0.12	22	27	1.17
1.3 M sucrose	9	44	0.56	9	37	0.20	20	40	1.17
2.0 M sucrose	6	26	0.20	8	30	0.09	13	19	0.36
unfractionated	100	100	0.09	100	100	0.05	100	100	0.50

[a] Data are expressed as percent distribution of glycosaminoglycans among the four subfractions from brain and liver microsomes. The concentration ratio was obtained by dividing the concentration of glycosaminoglycan in liver microsomes by the concentration in the corresponding subfraction from brain (concn ratio L/B). [b] Sucrose concentrations are those corresponding to the layers used to form the gradient. Actual sucrose molarities at which the various classes of membranes sedimented are somewhat lower (see Table I).

TABLE IV: Change in Distribution of Glycosaminoglycans (GAG) after Washing of Microsomal Membranes.

	hyaluronic acid (%)	chondroitin sulfate (%)	heparan sulfate (%)
% of total microsomal GAG present in subfraction 1	60	67	29
subfraction 1 GAG which resediments at 140 000*g*	2	3	10
% of total microsomal GAG removed by washing unfractionated microsomes	28	55	21
% of total "soluble" GAG[a] present in microsomal subfraction 1	59	32	50

[a] Total "soluble" GAG is the sum of the glycosaminoglycan present in the 140 000*g* supernatant of whole brain and that in microsomal subfraction 1.

in both brain and liver (e.g., as a component of cell surface or internal membranes), while hyaluronic acid and chondroitin sulfate are associated with cellular structures and functions more peculiar to nervous tissue. This difference between brain and liver is especially marked in the case of chondroitin sulfate, which is the major glycosaminoglycan of nervous tissue whereas even the small amounts present in liver appear to be of extrahepatic origin (Oldberg et al., 1977).

As summarized in Table IV, it was found that 60 and 67% of the total microsomal hyaluronic acid and chondroitin sulfate in brain are associated with the membranes of subfraction 1, whereas only 29% of the microsomal heparan sulfate is in this low density subfraction. When subfraction 1 was diluted to approximately 0.25 M sucrose and the membranes resedimented for 2 h at 140 000*g*, only 2–3% of the hyaluronic acid and chondroitin sulfate in this subfraction was recovered in the "washed" membranes, although 35% of the protein and glycoprotein hexosamine resediments under these conditions. Immediate washing of the unfractionated microsomes without allowing them to stand overnight on a density gradient at 4 °C removed somewhat lesser but still significant amounts of glycosaminoglycans (Table IV). However, a comparison of the amount of hyaluronic acid removed by washing the unfractionated microsomes (28%) with the percentage found in subfraction 1 and ultimately in a soluble form (60%) shows that a considerably smaller proportion of this glycosaminoglycan can be removed from the unfractionated microsomes than can be obtained from subfraction 1.

In contrast to the membranes of subfraction 1, less than 10% of the glycosaminoglycans were removed by washing of subfraction 2, and only traces from the higher density membranes, indicating that in these subfractions the glycosaminoglycans (and especially heparan sulfate) are integral constituents of the membranes.

The nature of the association of glycosaminoglycans with subfraction 1 membranes is not clear, but it is apparent that they must, at least initially, be avidly bound to the membranes, and that they do not represent soluble glycosaminoglycans which are merely occluded in the pellet of unfractionated microsomes. This conclusion is evident from the proportion of the total "soluble" glycosaminoglycans (i.e., the sum of that present in the 140 000*g* supernatant of whole brain and that found in microsomal subfraction 1) which is associated with subfraction 1 membranes, since 32 to 59% of the potentially soluble chondroitin sulfate, heparan sulfate, and hyaluronic acid is originally sedimented with the microsomes (Table IV), although the microsomal pellet occupies less than 2% of the volume of the 140 000*g* supernatant when measured with tritiated water.

TABLE V: Specific Activities of Soluble Glycosaminoglycans and Glycoproteins in Microsomal Subfraction 1 and in the 140 000*g* Supernatant of Whole Brain.[a]

	subfraction 1	140 000*g* supernatant
hyaluronic acid	6 000	6 700
heparan sulfate	27 600	24 900
chondroitin sulfate	16 800	19 200
glycoprotein hexosamine	20 300	26 300
glycoprotein sialic acid	11 900	22 700

[a] Specific activities determined after labeling for 48 h with [^{3}H]-glucosamine and expressed as cpm/μmol hexosamine or sialic acid.

To study this phenomenon, the binding capacity of subfraction 1 membranes for proteoglycans and glycoproteins was examined. For this purpose, rat brain glycosaminoglycans and glycoproteins were labeled in vivo by administration of [^{3}H]glucosamine, and after 2 days the 140 000*g* supernatant and the soluble portion of subfraction 1 were obtained by the usual methods. The whole brain soluble fraction was dialyzed overnight to remove free [^{3}H]glucosamine, and both soluble fractions (i.e., from whole brain and subfraction 1 membranes) were made 0.3 M in sucrose. Unlabeled brains were then homogenized in the two types of soluble extracts and microsomal subfractions were prepared separately from each by the usual procedure. It should be noted that the protein concentration of the "wash" derived from the subfraction 1 membranes was less than 10% of that present in the 140 000*g* supernatant.

In spite of this lower protein concentration, brains homogenized in subfraction 1 supernatant had 95% more protein in the second subfraction 1 when compared with tissue homogenized in 140 000*g* supernatant from whole brain. The absolute amount of this additional protein was almost identical with the total protein content of the subfraction 1 supernatant used for homogenization, but the available data do not permit us to conclude with certainty that there was a quantitative binding of this soluble protein to the membranes of the second subfraction 1. In spite of the large increase in *protein* concentration, there was very little binding of [^{3}H]glucosamine-labeled glycosaminoglycans and glycoproteins in either experiment (1.1% of the homogenate radioactivity for brains homogenized in whole brain supernatant and 1.8% of the soluble subfraction 1 radioactivity). Even though approximately two-thirds of the label in these soluble fractions was in glycoprotein hexosamine and sialic acid residues, any significantly increased binding of glycosaminoglycans from subfraction 1 as compared with those from a whole brain supernatant would have been evident from these studies had it occurred.

In an attempt to obtain further information concerning the possible relationships between the "soluble" glycosaminoglycans and glycoproteins of microsomal subfraction 1 and those isolated from the soluble fraction of whole brain, the specific activities of these complex carbohydrates were compared in the two fractions after 48 h of labeling with [^{3}H]-glucosamine in vivo. From the data summarized in Table V it can be seen that the specific activities of all three glycosaminoglycans are very similar (±7%) in both microsomal subfraction 1 and in the soluble fraction of whole brain, suggesting that the glycosaminoglycans in both fractions may be closely related metabolically. On the other hand, there were considerable differences in the specific activities of glycoprotein hexosamine and sialic acid in these two fractions, which precludes their both originating from the same metabolic pool.

Time Course of Labeling of Glycosaminoglycans and

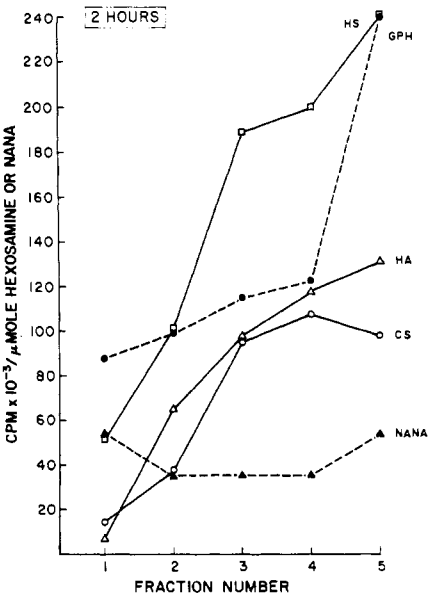

FIGURE 3: Specific activities of glycosaminoglycans and glycoproteins in microsomal subfractions of brain 2 h after administration of [^{3}H]glucosamine. (○) Chondroitin sulfate; (△) hyaluronic acid; (□) heparan sulfate; (●) glycoprotein hexosamine; (▲) glycoprotein sialic acid.

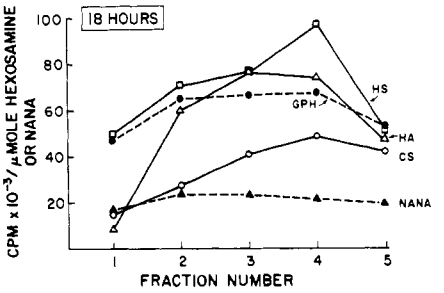

FIGURE 4: Same as Figure 3 for 18 h after administration of [^{3}H]glucosamine.

Glycoproteins in Microsomal Subfractions. The specific activities of hyaluronic acid, chondroitin sulfate, heparan sulfate, and glycoprotein hexosamine and sialic acid were determined in the microsomal subfractions at four time intervals after administration of [^{3}H]glucosamine (2 h, 18 h, 48 h, and 8 days). The results of these studies are illustrated in Figures 3 to 6. Maximum specific activities of hyaluronic acid, heparan sulfate, and glycoprotein hexosamine are observed in the rough endoplasmic reticulum by 2 h, while at this time maximally labeled chondroitin sulfate is present in the next lower density subfraction, where high specific activity heparan sulfate, hyaluronic acid, and glycoproteins are also found 18 h after labeling. There is then a progressive movement of these newly synthesized complex carbohydrates to still lower density subfractions consisting predominantly of plasma membranes, smooth endoplasmic reticulum, and golgi complex. Because the dose and absorption of labeled glucosamine varied somewhat from one experiment to another, it is possible to make meaningful comparisons of absolute specific activities only within but not between the different experiments, though relative changes in the labeling of complex carbohydrates can be compared at different time points. It should also be noted that, since brain glycosaminoglycans and glycoproteins have relatively long turnover half-times (Margolis & Margolis, 1973, 1977), one can assume that changes in their specific activities between 2 h and 2 days are largely attributable to

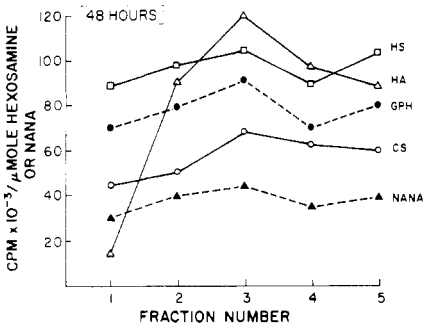

FIGURE 5: Same as Figure 3 for 48 h after administration of [^{3}H]glucosamine.

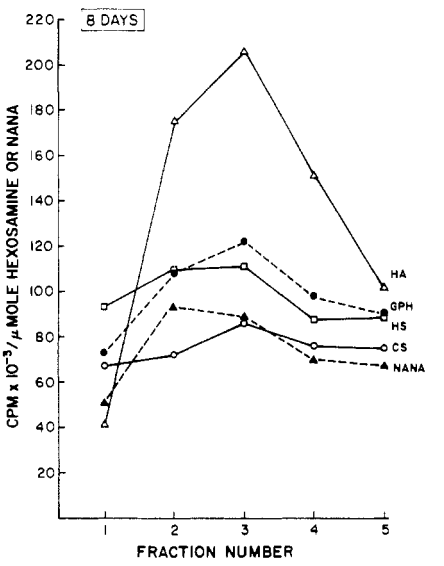

FIGURE 6: Same as Figure 3 for 8 days after administration of [^{3}H]glucosamine.

movement of complex carbohydrates between the various membrane subfractions.

The finding that hyaluronic acid specific activity is highest in the fraction enriched in rough endoplasmic reticulum after 2 h of labeling suggests that its biosynthesis is also initiated at this site in a manner similar to that of the glycoproteins and sulfated glycosaminoglycans, even though there is no conclusive evidence that hyaluronic acid is covalently linked to protein as are these other complex carbohydrates. The unexpectedly high specific activity of glycoprotein sialic acid (Figure 3) and of gangliosides (unpublished results) in subfraction 5 raises the further possibility that components of the rough endoplasmic reticulum may contain significant amounts of sialyl and other glycosyl transferases which are also involved in later stages of glycoprotein and glycolipid biosynthesis.

These results cannot be due to contamination of subfraction 5 with golgi membranes or smooth endoplasmic reticulum, since no other subfraction had a higher specific activity of hyaluronic acid, glycoprotein sialic acid, or gangliosides at this early time point. The relatively high specific activity of the glycoproteins and sulfated glycosaminoglycans in subfraction 5 even as long as 8 days after administration of [^{3}H]glucosamine is, however, probably attributable to the presence in this fraction of membranes derived from other sources (e.g., mitochondria, lysosomes) than rough endoplasmic reticulum.

It was also found that at 8 days after labeling, hyaluronic acid attains a very high specific activity in microsomal subfraction 3 (Figure 6). Since we have previously demonstrated the presence in brain of hyaluronate pools with turnover half-times of 9 and 45 days (Margolis & Margolis, 1973), it is possible that the membranes of subfraction 3 are associated with the hyaluronate pool having the slower turnover rate. Very little of the newly synthesized hyaluronic acid would have been removed from this pool by 8 days, whereas almost half of the labeled hyaluronate would have disappeared from the rapid turnover pool.

Discussion

Although detailed information concerning the localization and specific functional roles of brain glycosaminoglycans and glycoproteins is not yet available, it is known that the glycoproteins are mostly present in plasma and intracellular membranes, where they are probably involved in various types of cell-cell interactions and as components of receptors for neurotransmitters, drugs and hormones (for reviews, see Margolis & Margolis, 1977, 1979).

In contrast to the glycoproteins, a large portion (approximately 40%) of the glycosaminoglycans occurs in brain in a soluble form. Based on our previous finding that the concentration of glycosaminoglycans in neurons and astrocytes is considerably greater than that in whole cerebra from which the cells were isolated (Margolis & Margolis, 1974), it appeared that the soluble pool of these complex carbohydrates is primarily cytoplasmic rather than extracellular, since it would otherwise have been lost during the cell isolation procedure. Their cytoplasmic localization has recently been directly demonstrated by studies using isolated neurons, from which much of the chondroitin sulfate and hyaluronic acid can be recovered, together with lactate dehydrogenase, in the cell-free supernatant following hypotonic lysis (unpublished results).

In an earlier study we found that most of the particulate glycosaminoglycans are present in the microsomal fraction of brain (Margolis et al., 1975). Beginning from this observation, we have now prepared subfractions of these membranes which were characterized with respect to morphology, enzyme composition, and the structure, distribution, and metabolism of glycosaminoglycans, glycoproteins, and gangliosides. Although reports have appeared concerning the protein and lipid composition of microsomal subfractions from brain (Got et al., 1967; Tamai et al., 1974), their complex carbohydrates have not previously been investigated.

Most of the microsomal hyaluronic acid and chondroitin sulfate was found in a low density fraction of smooth membranes which are probably derived from both plasma membranes and smooth endoplasmic reticulum. The concentration of lipid in this subfraction would appear to be the same as or slightly less than average, based on the percentage of lipid-free protein residue in the dialyzed, lyophilized membranes. This indicates that their low density is not due to a greater proportion of lipid, but rather to the fact that they form large vesicles containing a sufficient amount of water to give them a low buoyant density. It is, however, not possible to determine the relative contributions to subfraction 1 of plasma membranes and smooth endoplasmic reticulum on the basis either of electron microscopy, or of enzyme activities (due to the somewhat unspecific nature of the currently used enzyme markers).

In both brain and liver a larger porportion of heparan sulfate than of hyaluronic acid and chondroitin sulfate is associated with the higher density microsomal membrane subfractions. This heparan sulfate was not removed by washing the membranes, which are also somewhat enriched in [Na^+-K^+]-

ATPase and have only half of the NADPH-cytochrome *c* reductase activity found in subfraction 1. Since we have previously demonstrated that synaptic plasma membranes do not contain significant amounts of glycosaminoglycans (Margolis et al., 1975), these data suggest that heparan sulfate may be a constituent of plasma membranes derived from nonsynaptic areas of the cell surface and are supported by histochemical evidence for the presence in neuronal membranes of a hyaluronidase-resistant polyanion resembling heparan sulfate in its staining characteristics with Alcian Blue (Castejón, 1970). The postulated localization of heparan sulfate in brain plasma membranes is also consistent with the conclusions from a number of biochemical studies indicating that heparan sulfate is present on the surface of a wide variety of cell types (Kraemer, 1971; Kraemer & Smith, 1974; Buonassisi & Root, 1975; Akasaki et al., 1975; Underhill & Keller, 1977; Oldberg et al., 1977). Attempts to study this question further using isolated neurons are currently in progress.

There have previously been several reports on the distribution and metabolism of sulfated macromolecules in the golgi complex, but since these have mostly concerned secretory tissues their applicability to brain is difficult to assess. Histochemical studies have provided evidence that glycosaminoglycans are present in the golgi complex of intestinal goblet cells (Berlin, 1967), and autoradiographic experiments have demonstrated the incorporation of labeled sulfate into macromolecular components in the golgi complex and zymogen granules of pancreatic exocrine cells in the mouse (Berg & Young, 1971). More recent studies have also demonstrated a selective distribution of anionic sites on the surfaces of different structural components of the rat liver golgi complex (Abe et al., 1976), and that there is a rapid incorporation of inorganic sulfate into rat liver golgi membranes (Katona, 1976). It may be significant that a large portion of the initially particulate sulfate radioactivity found in liver golgi complex after a short period of labeling (15 min) was solubilized during further purification of the golgi membranes on a second sucrose gradient (Katona, 1976), a situation similar to that which we observed in the case of microsomal membranes from brain.

In view of these reports it is of interest that the brain microsomal subfractions enriched in golgi membranes have a relatively low concentration of glycosaminoglycans, and analysis of rat liver golgi membranes indicates that the anionic materials present there are more likely sialoglycoproteins than glycosaminoglycans (Margolis & Moscarello, unpublished results). These findings do not, however, exclude the possibility that the golgi complex may play an important role in glycosaminoglycan biosynthesis, even though the pool of completed product is relatively small.

It is well known that microsomes have the ability to adsorb basic proteins on the negatively charged surface of endoplasmic reticulum fragments, and that various secretory proteins present in the lumen of the intact endoplasmic reticulum can become trapped in microsomal vesicles produced during the process of homogenization (DePierre & Dallner, 1976). We do not, however, believe that the glycosaminoglycans found associated with microsomal subfraction 1 represent an instance of this type of nonspecific binding. Based on our previous analysis of the distribution of glycosaminoglycans in subcellular fractions obtained by differential centrifugation of a brain homogenate (Margolis et al., 1975), and later results demonstrating only very small amounts in purified nuclei (Margolis et al., 1976), it can be concluded that at least 40 to 60% of the chondroitin sulfate and hyaluronic acid of rat brain is associated with microsomal membranes. The large proportion of these two brain glycosaminoglycans which is recovered almost entirely in a single microsomal subfraction argues against a nonspecific adsorption or binding. Their acidic character also makes it unlikely that they would complex with the negatively charged sites known to be present on the surface of the endoplasmic reticulum (Dallner & Azzi, 1972), and such adsorbed proteins are removed very poorly or not at all by washing with sucrose (as compared with salt solutions), whereas the glycosaminoglycans are easily removed by washing with sucrose.

It has been demonstrated that sulfated glycosaminoglycans such as chondroitin sulfate and heparin are synthesized in the endoplasmic reticulum, where polymerization of the polysaccharide chains takes place with microsomal enzymes on previously formed microsomal proteoglycan primer (Horwitz & Dorfman, 1968; Richmond et al., 1973; DeLuca et al., 1973; Lindahl et al., 1977). The results of our studies on the time course of labeling of chondroitin sulfate and heparan sulfate in microsomal subfractions of brain are consistent with this general scheme. Although the mechanism of hyaluronic acid biosynthesis in animal tissues is poorly understood, our findings suggest that the biosynthetic sites and pathways for hyaluronic acid may be very similar to those for the sulfated glycosaminoglycans, even though the possible role of a peptide primer remains uncertain.

The membranes of microsomal subfraction 1 appear to be a mixed population derived from both the cell surface and the smooth endoplasmic reticulum. The expected similarities in composition and the probable biosynthetic relationships between these two types of membranes are discussed in the following paper in connection with a comparison of the glycoprotein composition of microsomal and synaptic membranes (Krusius et al., 1978). The results of our studies do not allow us to draw definitive conclusions concerning the exact localization of the particulate glycosaminoglycans of brain, but it would appear that hyaluronic acid and chondroitin sulfate are associated with a different type of membrane than is heparan sulfate. In brain most of the heparan sulfate is firmly bound to membranes of average density, from which it cannot be dissociated by washing with sucrose or salt solutions. These properties are consistent with a localization in plasma membranes similar to that reported for a number of other tissues as discussed above. It is possible that the particulate hyaluronic acid and chondroitin sulfate are also largely present in plasma membranes and/or as a cell coating which extends into the extracellular space. However, any conclusive statement concerning this problem will require confirmatory cytochemical or other evidence pertaining to the localization of these glycosaminoglycans in situ, since it is not presently possible to prepare purified plasma membranes of nonsynaptic origin at an age when developmental changes in the levels and distribution of brain glycosaminoglycans have ceased to occur.

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