

Glycosphingolipids from cultured astroblasts

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Abstract The glycolipids of two clonal lines of astroblasts, NN clone and C6 clone, were studied. Glucosylceramide and lactosylceramide were present at very low levels in both clones, but the most common myelin glycolipids, galactosylceramide and sulfatide, were not detected. The ganglioside pattern of these cells was rather simple, with G_{M3} as main component, accompanied in one clone by G_{D3} . These results are quite different from those observed on astroglia-enriched fractions isolated from brain. The fatty acid composition of these glycolipids was studied. Long chain fatty acids, up to lignoceric acid, were found in appreciable amounts, even in gangliosides. It is difficult to conclude if these glycolipid patterns are due to the properties of normal astroblasts, to transformation, or to a tumoral character of our cell lines.

Supplementary key words fatty acid compositions · gangliosides · glial cells · glucosylceramide · lactosylceramide

Although the lipid composition of the whole brain is actually well known, the distribution of these lipids among the various types of cells present in brain remains an area of considerable investigation. The usual way to answer this question is the analysis of bulk neurons and glia isolated from brain by centrifugation procedures (see review by Norton et al. (1)). Another way of approaching this problem is to analyze the lipids of cultured cells derived from brain tissue. Neuroblastoma cells could be considered as a model for neurons, and several studies have been devoted to their lipids, mainly their glycosphingolipids (2, 3). Glial cell lines should also be of great interest to approach the lipid composition of glial cells in situ. We have already reported the distribution of neutral lipids and phospholipids of two astroblast cell lines (4); we report here their glycosphingolipid compositions. A part of this work has previously been presented (5).

MATERIALS AND METHODS

Cell culture

Normal astroblasts originated from clone NN (normal baby hamster astroglia) isolated by Shein et al.

(6). Tumorous rat astroblasts originated from clone C6 isolated by Benda et al. (7). The cells were cultivated in Falcon dishes with Eagle-Dulbecco medium supplemented with 10% fetal calf serum (GIBCO). The cells were harvested at the beginning of the stationary phase. They were recovered by scraping, washed in 0.9% NaCl, and freeze-dried.

Lipid extraction and fractionation

The lipids were extracted from lyophilized cells by the technique of Folch, Lees, and Sloane Stanley (8) with the modification of Suzuki (9). Three homogenizations in large amounts (approximately 1000 ml per g dry weight) of chloroform-methanol 2:1 (v/v) containing 5% water, and two subsequent homogenizations in chloroform-methanol 1:2 (v/v) were performed. After reequilibrating the chloroform-methanol ratio to 2:1 (v/v), 0.2 vol of 0.88 M KCl was added; the mixture was shaken and the two phases were allowed to separate.

The upper phase was removed and the lower one was washed two times with a theoretical upper phase (chloroform-methanol-water 3:48:47, v/v/v). The combined upper phases were concentrated to a small volume under reduced pressure and dialyzed for 2 days against distilled water. No loss of gangliosides occurred during this dialysis. After dialysis, the dialyzed material was lyophilized and then dissolved in a small volume of chloroform-methanol 2:1 (v/v) for the study of gangliosides.

The lower phase was evaporated to dryness under vacuum and fractionated by silicic acid column chromatography according to Vance and Sweeley (10) by successive elutions with chloroform, acetone, and methanol. The acetone fraction was the only one containing lipid hexoses; the phospholipids present in this fraction represented less than 5% of the

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; LCB, long-chain bases. The ganglioside nomenclature of Svennerholm (Reference 34) is used throughout. Fatty acids are abbreviated in the usual manner, i.e., a number indicating chain length followed by the number of double bonds per molecule.

total cell phospholipids. Trace amounts of G_{M3} ganglioside were found in this glycolipid fraction but accounted for no more than 5% of the G_{M3} of the corresponding ganglioside fraction.

Assays of lipid hexose and sialic acids

Lipid hexose from the lower phase was assayed according to Svennerholm (11). Sialic acid from the upper phase was assayed by the method of Svennerholm (12) with the color extraction of Miettinen and Takki-Luukkainen (13).

Analytical thin-layer chromatography of glycolipids

The glycolipids of the lower phase were separated on precoated silica gel plates, 0.25 mm thick (Merck). The solvent was chloroform–methanol–water 100:42:6 (v/v/v) (14). The spots were visualized with the orcinol– H_2SO_4 reagent of Svennerholm (11) and the glycolipids were quantitated by densitometry (15) by comparison with lipid standards (2–20 μg) chromatographed on the same plate. The values found by this technique were in good agreement with those predicted from the lipid hexose assay, assuming that the identification of the spots was correct. Borate-impregnated plates were also used for a tentative separation of monohexosylceramides (15).

The gangliosides were separated on the same type of plates, with the solvent described by Van den Eijnden (16) (chloroform–methanol–water 60:35:8 (v/v/v), containing 20 mg of KCl per 100 ml). The spots were visualized with Bial's orcinol–HCl reagent (17). The distribution of sialic acid among the gangliosides was done by densitometry.

Preparative TLC of glycolipids

Glass plates of 20 × 20 cm were coated with 1 mm of silica gel H containing 10% Florisil. The same solvents as those used for analytical TLC were used. Lipids were visualized by spraying distilled water on the plate. The parts of the silica gel that contained lipids were then poured on small silicic acid columns and eluted with chloroform–methanol–water 50:50:15 (v/v/v) after a washing with pure chloroform.

Structural study of glycolipids

Each glycosphingolipid was submitted to an acidic methanolysis using the technique of Zanetta, Breckenridge, and Vincendon (18). The fatty acid methyl esters were extracted three times with hexane and the remaining sugars and long chain bases (LCB) were submitted to a trifluoroacetylation according to Zanetta et al. (18).

The fatty acid methyl esters were purified by TLC using silica gel G plates washed by the technique

of Carreau, Lapous, and Raulin (19). Spots corresponding to hydroxy fatty acids could never be detected, even after carbonization of the lipids on the plate. The fatty acid methyl esters were eluted with chloroform, and dissolved after evaporation in a small volume of hexane. GLC of fatty acid methyl esters was performed at 180°C using a glass column packed with EGSS-X (10% on Chromosorb W-HP as a support). The fatty acids were identified by comparison with standards and by the use of semi-logarithmic plotting.

The sugars and LCB were chromatographed, after trifluoroacetylation, on a glass column packed with OV 210 (3% on Chromosorb W-HP) using a temperature program of 120–200°C according to Zanetta et al. (18). The identification of sugars was easy to make, but a lack of appropriate standards did not allow us to identify the LCB, the peaks of which appeared between 190 and 200°C. The relative molar responses used to calculate the molar ratios of the sugars were mesoinositol (internal standard), 1000; galactose, 945; glucose, 945; *N*-acetylneuraminic acid, 1100.

For both sugars and LCB we measured the areas of the peaks by multiplying the height by the width at half-height. All chromatographic analyses were carried out on a Varian Aerograph gas chromatograph, using a flame ionization detector.

Standard lipids

Glucosylceramide and lactosylceramide were generous gifts of Dr. L. Sarliève; ganglioside standards were prepared by Mrs. P. Guérin; fatty acid standards were purchased from Fluka. All chemicals and solvents were of the highest purity available commercially.

RESULTS

Neutral glycolipids

In both glial cell lines only two neutral glycolipids could be detected after TLC (**Fig. 1**), even when using borate-impregnated plates. They ran, respectively, like standards of glucosylceramide and lactosylceramide. The GLC of the sugars of the isolated lipids showed that the fast-moving spots contained only glucose; the slow-moving spots contained equal amounts of glucose and galactose.

The concentrations of these two lipids in our cell lines were very low (**Table 1**). The amounts of glucosylceramide were similar in both clones but lactosylceramide was about 20 times more abundant

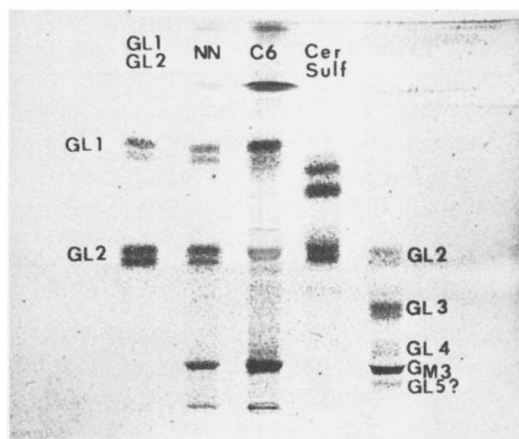


Fig. 1. TLC separation of the neutral glycolipids of NN and C6 cells. The silica gel plate 0.25 mm thick was first impregnated with anhydrous sodium tetraborate (15) before spotting the lipids. It was then developed 2 hr with chloroform-methanol-water 100:42:6 (v/v/v). The spots were detected by spraying with the orcinol-H₂SO₄ reagent and heating at 140°C for 15 min. From left to right, the materials chromatographed were lane 1, mixture of glucosylceramide (GL1) and lactosylceramide (GL2) from pig kidney; lane 2, glycolipids from NN cells; lane 3, glycolipids from C6 cells; lane 4, mixture of galactosylceramide (Cer) and sulfatide (Sulf) from pig brain; lane 5, mixture of di-, tri-, tetra-, penta-hexosylceramide and G_{M3} ganglioside from pig kidney (respectively GL2, GL3, GL4, GL5 and G_{M3}).

in NN cells than in C6 cells. We could never detect appreciable amounts of the most important galactolipids of brain tissue, galactosylceramide (cerebroside) and sulfatide, and we assumed the absence of sulfatide on the basis of GLC results. However, it should be pointed out that the methodology would not permit the detection of these lipids if they constituted less than approximately 5% of the GL-2 spot.

The fatty acid compositions of the lipids are shown in **Table 2**. No hydroxy fatty acids could be found, but very long chain fatty acids (length chain 22 or 24 carbons) were present in a rather high amount. The NN clone contained more long chain fatty acids and more unsaturated fatty acids than the C6 clone. The lactosylceramides of both clones were richer in short chain fatty acids than glucosylceramides; a higher level of unsaturated fatty acids was found in glucosylceramide in NN clone and in lactosylceramides in C6 clone.

TABLE 1. Amounts of neutral glycolipids in NN and C6 cells

	NN cells	C6 cells
	<i>μg lipid per mg dry weight</i>	
Glucosylceramide	0.23 ± 0.02 ^a	0.16 ± 0.02
Lactosylceramide	1.01 ± 0.03	0.05 ± 0.01

^a Each value is the mean of four experiments ±SD.

TABLE 2. Fatty acid distributions of glucosylceramide (GL1) and lactosylceramide (GL2) of NN and C6 cells

	NN cells		C6 cells	
	GL1	GL2	GL1	GL2
	<i>% by weight</i>			
14:0	0.3 ^a	1.1	2.9	4.1
15:0	0.3	1.0	1.7	4.2
16:0	10.0	30.7	21.7	33.9
16:1	0.8	3.3	1.7	5.2
18:0	3.4	9.3	14.0	19.1
18:1	13.1	3.8	6.2	10.3
19:0	0.2			
19:1	0.8	0.3	0.9	1.5
20:0	2.0	2.5	3.3	1.0
20:1	1.2	0.2		0.5
21:0	4.5			
22:0	13.4	12.9	8.5	2.3
22:1	13.9	1.3		0.6
23:0	2.6	1.8		
24:0	15.3	16.2	31.4	11.2
24:1	14.5	15.6	7.7	5.0
>24				
>20	67.9	47.8	47.6	19.1
Unsat.	44.3	24.5	16.5	23.1

^a Each value is the mean of three experiments.

Gangliosides

The main ganglioside present in appreciable amounts in C6 clone was G_{M3} (**Fig. 2**). This lipid was identified by TLC and its sugar moiety was identified by GLC. The molar ratio of glucose/galactose/sialic acid was 1.0/0.9/1.0. The only sialic acid found was *N*-acetyl neuraminic acid. We could never detect *N*-glycolyl neuroaminic acid.

NN clone contained two main gangliosides, which migrated like G_{M3} and G_{D3} (**Fig. 2**). The molar ratios glucose/galactose/sialic acid obtained after GLC were 1.0/0.9/1.1 for the upper spot and 1.0/0.9/1.9 for the lower one. These results confirmed the TLC identification of the lipids. *N*-Glycolyl neuraminic acid was never detected.

Each ganglioside migrated on TLC as two spots very close to each other. We failed to detect any difference in sugar composition between these spots, but it seems that the fast-moving one was enriched in long chain fatty acids and the slow-moving one was enriched in short chain fatty acids. A similar phenomenon is well known for sphingomyelin (20). Trace amounts of other gangliosides were found on thin-layer plates; they migrated like standards of G_{M2}, G_{M1} and G_{D1a} but their levels were too low to obtain their compositions or their fatty acid distributions.

The amount of gangliosides was very low in both clones (about 0.5 μg of lipid sialic acid per mg dry weight). The distributions of NN and C6 gangliosides are presented in **Table 3**.

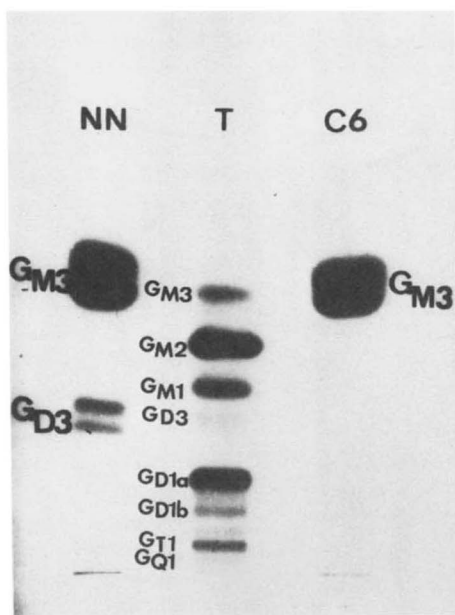


Fig. 2. TLC separation of the gangliosides of NN cells (left) and C6 cells (right). A mixture of gangliosides from pig brain G_{M3} and Tay-Sachs ganglioside (G_{M2}) ran in the middle. Samples were spotted on a silica gel plate 0.25 mm thick (Merck). The plate was developed 6 hr in chloroform-methanol-water 60:35:8 (v/v/v) containing 20 mg KCl per 100 ml solvent, and the spots were detected by spraying with the orcinol-HCl reagent, covering the plate with glass, and heating at 140°C for 15 min.

The fatty acid distributions of gangliosides from both clones are shown in **Table 4**. These patterns were different for several fatty acids. However, palmitic acid and lignoceric acid were always the most abundant fatty acids. The presence of high amounts of long chain fatty acids must be emphasized since these acids are rarely found in gangliosides.

DISCUSSION

Compared to the astrocyte fractions isolated from brain (21–23), NN or C6 astroblasts present strikingly different patterns of glycosphingolipids. The pres-

TABLE 3. Amounts of gangliosides in NN and C6 cells

	NN cells	C6 cells
	$\mu\text{g lipid per mg dry weight}$	
Total gangliosides	2.4 ± 0.2^b	2.0 ± 0.2
G_{M3}^a	66.8 ± 3.3	95.2 ± 3.2
G_{M2}		1.8 ± 0.2
G_{M1}	1.3 ± 0.2	
G_{D3}	30.6 ± 2.1	2.3 ± 0.3
G_{D1a}	2.3 ± 0.2	0.7 ± 0.2

^a Individual gangliosides are expressed as percentage of total lipid sialic acid.

^b Each value is the mean of 10 experiments \pm SD for NN cells and 5 experiments \pm SD for C6 cells.

TABLE 4. Fatty acid distributions of gangliosides of NN and C6 cells

	NN cells		C6 cells
	G_{M3}	G_{D3}	G_{M3}
	% by weight		
14:0	0.4 ^a		0.4
15:0	0.3		0.3
16:0	25.1	20.6	17.3
16:1	0.4	0.7	0.9
17:0	0.5	1.6	1.2
18:0	3.1	16.1	10.0
18:1	0.5	4.6	1.8
19:0	0.1	0.5	0.3
20:0	4.1	4.2	3.4
20:1			
21:0	0.2		
22:0	21.2	12.8	9.3
22:1	0.1	0.6	0.4
23:0	2.8	1.9	1.8
24:0	33.9	26.4	29.6
24:1	7.3	10.0	18.7
>24			4.6
>20	65.5	51.7	64.4
Unsat.	8.3	15.9	21.8

^a Each value is the mean of three experiments.

ence of galactosylceramide and sulfatide in astrocyte fractions was reported by Norton and Poduslo (22), Hamberger and Svennerholm (21), Raghavan and Kanfer (24), and Abe and Norton (23). We failed to detect the presence of these lipids in our cells. This discrepancy between astroglia isolated from brain and cultured astroglia could be explained by a contamination of the glial fractions by a galactolipid-rich subfraction of brain, i.e., myelin. Norton and Poduslo (22) showed that up to 30% contamination of nonastrocytic material was present in their glial fractions. As myelin contained, even in young rat brains, 30–40 times more galactosylceramide or sulfatide than glial fraction (in comparison to dry weights) (23), a contamination of 3% of the glial fraction with myelin fragments would be enough to account for the levels of galactolipids in astroglia found by Abe and Norton (23), Hamberger and Svennerholm (21), or Norton and Poduslo (22).

The presence of glucosylceramide and lactosylceramide in our cells is in good agreement with the data of Abe and Norton (23) who reported the presence of these lipids in very low concentrations in astrocytes and neurons from immature rat brains, and their absence in myelin. These lipids are also found in neuroblastoma cells (2, 3). Thus, these minor components of brain lipids (25) should be located in membranes of both astrocytes and neurons rather than in myelin.

The fatty acid compositions of glucosylceramide and lactosylceramide of our cells agree with the data presented for brain (25) or cellular fractions

of brain (23). However, we found in every case higher amounts of long chain fatty acids and lower amounts of stearic acid.

The very simple ganglioside pattern found in our cells is strikingly different from all the data published concerning astrocytes isolated from brain (5, 21, 23). However, Derry and Wolfe (26) found such low amounts of gangliosides in glial cells isolated by microdissection techniques. The low levels of gangliosides we found and their simple structures were observed generally in nonneural tissues. The problem has not yet been solved whether or not astrocytes did contain the large amounts of complex gangliosides that were found by authors who isolate glial cells by differential centrifugation techniques (5, 21–23). In view of the large amounts of gangliosides present in synaptosomal plasma membranes (27), a slight contamination of the astrocyte fraction by synaptosomes could explain these results. Such contaminations have been shown to occur by several authors (28–30). Nevertheless, one must keep in mind that cultured cell lines always present an embryonic or even a tumorous character that can affect strongly the ganglioside profile. Several authors have shown that the malignant transformation of a cell is accompanied by a decrease in glycolipid concentration and by a simplification of the structure of these glycolipids (for review see 31 and 32). Such a phenomenon may have occurred for C6 cells that have a tumorous origin. NN cells, which grow in continuous lines, may have undergone a transformation; however, the rat astroblasts in primary cultures we have studied in a previous work (5) present a ganglioside pattern similar to that of C6 clone. Thus it seems that no definitive conclusion can be made about the presence or the absence of large amounts of complex gangliosides in astroglia.

The fatty acid distribution of the gangliosides of our cell lines is quite different from the usual distribution found in gangliosides from brain (33) or its cellular fractions (23). Long chain fatty acids are found normally in neutral glycolipids but not in gangliosides from brain. Their presence in the gangliosides of our cells may be caused by the fact that they are cultured with serum, which contains long chain fatty acids.

It must be pointed out that Dawson et al. (2) found in C6 cells the two gangliosides G_{M3} and G_{D3} , at a ratio close to that we found for NN cells. The lack of G_{D3} in our C6 cells could be explained by the progressive modifications that tumorous cells undergo (31, 32). ■■

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REFERENCES

1. Norton, W. T., T. Abe, S. E. Poduslo, and G. DeVries. 1975. The lipid composition of isolated brain cells and axones. *J. Neurosci. Res.* **1**: 57–75.
2. Dawson, G., S. F. Kemp, A. C. Stoolmiller, and A. Dorfman. 1971. Biosynthesis of glycosphingolipids by mouse neuroblastoma (NB41A), rat glia (RGC-6) and human glia (CHB-4) in cell culture. *Biochem. Biophys. Res. Comm.* **44**: 687–694.
3. Yogeewaran, G., R. K. Murray, M. L. Pearson, B. D. Sanwal, F. A. McMorris, and F. H. Ruddle. 1973. Glycosphingolipids of clonal lines of mouse neuroblastoma and neuroblastoma XL cell hybrids. *J. Biol. Chem.* **248**: 1231–1239.
4. Robert, J., P. Mandel, and G. Rebel. 1976. Neutral lipids and phospholipids from cultured astroblasts. *J. Neurochem.* **26**: 771–777.
5. Robert, J., L. Freysz, M. Sensenbrenner, P. Mandel, and G. Rebel. 1975. Gangliosides of glial cells: a comparative study of normal astroblasts in tissue culture and glial cells isolated on sucrose–Ficoll gradients. *FEBS Lett.* **50**: 144–146.
6. Shein, H. M., A. Britva, H. H. Hess, and D. J. Selkoe. 1970. Isolation of hamster brain astroglia by in vitro cultivation and subcutaneous growth, and content of cerebroside, ganglioside, RNA and DNA. *Brain Res.* **19**: 497–501.
7. Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differentiated rat glial cell strain in tissue culture. *Science* **161**: 370–371.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497–509.
9. Suzuki, K. 1964. A simple and accurate micromethod for quantitative determination of ganglioside patterns. *Life Sci.* **3**: 1227–1233.
10. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of the neutral glycosylceramides in human blood. *J. Lipid Res.* **8**: 621–630.
11. Svennerholm, L. 1956. The quantitative determination of cerebroside in nervous tissue. *J. Neurochem.* **1**: 42–53.
12. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol–hydrochloric acid method. *Biochim. Biophys. Acta.* **24**: 604–611.
13. Miettinen, T., and I. T. Takki-Luukkainen. 1959. Use of butyl acetate in determination of sialic acid. *Acta Chem. Scand.* **13**: 856–858.
14. Esselman, W. J., J. R. Ackermann, and C. C. Sweeley. 1973. Glycosphingolipids of membrane fractions from normal and transplanted canine kidney. *J. Biol. Chem.* **248**: 7310–7317.
15. Robert, J., and G. Rebel. 1975. Quantitation of neutral glycolipids by thin-layer chromatography on pre-coated plates. *J. Chromatogr.* **110**: 393–397.
16. Van den Eijnden, D. N. 1971. Chromatographic separation of gangliosides on precoated silica gel thin-layer plates. *Hoppe Seyler's Z. Physiol. Chem.* **352**: 1601–1602.
17. Klenk, E., and W. Gielen. 1961. Untersuchungen über die Konstitution der Ganglioside aus Menschenhirn und die Trennung des Gemisches in die

Komponenten. *Hoppe Seyler's Z. Physiol. Chem.* **326**: 144–157.

18. Zanetta, J. P., W. C. Breckenridge, and G. Vincendon. 1972. Analysis of monosaccharides by gas-liquid chromatography of the *O*-methyl glycosides as trifluoroacetate derivatives. *J. Chromatogr.* **69**: 291–304.
19. Carreau, J. P., D. Lapous, and J. Raulin. 1969. Artéfacts expérimentaux pouvant survenir lors de la chromatographie en couche mince des lipides membranaires. *J. Chromatogr.* **42**: 422–425.
20. Lastennet, A., L. Freysz, and P. Mandel. 1972. Hétérogénéité métabolique des sphingomyélines du cerveau de rat. *J. Neurochem.* **19**: 831–840.
21. Hamberger, A., and L. Svennerholm. 1971. Composition of gangliosides and phospholipids of neuronal and glial cells enriched fractions. *J. Neurochem.* **18**: 1821–1829.
22. Norton, W. T., and S. E. Poduslo. 1971. Neuronal perikarya and astroglia of rat brain: chemical composition during myelination. *J. Lipid Res.* **12**: 84–90.
23. Abe, T., and W. T. Norton. 1974. The characterization of sphingolipids from neurons and astroglia of immature rat brain. *J. Neurochem.* **23**: 1025–1036.
24. Raghavan, S., and J. N. Kanfer. 1972. Ceramide galactoside of enriched neuronal and glial fractions from rat brain. *J. Biol. Chem.* **247**: 1055–1056.
25. Hammarström, S. 1971. Brain glucosyl ceramide containing 2-hydroxy fatty acids. Identification of molecular species by gas-liquid chromatography-mass spectrometry. *Eur. J. Biochem.* **21**: 388–392.
26. Derry, D. M., and L. S. Wolfe. 1967. Gangliosides in isolated neuron and glial cells. *Science.* **158**: 1450–1452.
27. Breckenridge, W. C., G. Gombos, and I. G. Morgan. 1972. The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochim. Biophys. Acta.* **266**: 695–707.
28. Cremer, J. E., P. V. Johnston, B. I. Roots, and A. J. Trevor. 1968. Heterogeneity of brain fractions containing neuronal and glial cells. *J. Neurochem.* **15**: 1361–1370.
29. Sinha, A. K., and S. P. R. Rose. 1971. Bulk separation of neurons and glia: a comparison of techniques. *Brain Res.* **33**: 205–217.
30. Hemminki, K., E. Hemminki, and E. Giacobini. 1973. Activity of enzymes related to neurotransmission in neuronal and glial fractions. *Intern. J. Neurosci.* **5**: 87–90.
31. Hakomori, S. I. 1975. Structures and organization of cell surface glycolipids dependency on cell growth and malignant transformation. *Biochim. Biophys. Acta.* **417**: 55–89.
32. Brady, R. O., and P. H. Fishman. 1974. Biosynthesis of glycolipids in virus-transformed cells. *Biochim. Biophys. Acta.* **355**: 121–148.
33. Rosenberg, A., and N. Stern. 1966. Changes in sphingosine and fatty acid components of the gangliosides in developing rat and human brain. *J. Lipid Res.* **7**: 122–131.
34. Svennerholm, L. 1964. The Gangliosides. *J. Lipid Res.* **5**: 145–155.