Novel Gangliosides of Frog Brain. Establishment of the Structures by their ^{1}H N.m.r. and Mass Spectra

Kiyotaka Munesada, Akira Nishikawa, and Takayuki Suga *
Department of Chemistry, Faculty of Science, Hiroshima University, Higashisenda-machi, Naka-ku, Hiroshima 730, Japan

The structures of three gangliosides isolated from bull frog (*Rana catesbeiana*) brain tissue have been established as disialosylgangliotetraosylceramide (III^{6}NeuAcIV^{3}NeuAc-GgOse$_4$Cer), trisialosylgangliotetraosylceramide [III^{6}NeuAcIV^3(NeuAc)$_2$-GgOse$_4$Cer], and tetrasialosylgangliotetraosylceramide [III^6(NeuAc)$_2$IV^3(NeuAc)$_2$-GgOse$_4$Cer] by ^{1}H n.m.r. and mass spectral measurements as well as by chemical methods. Although the carbohydrate entity in disialosylgangliotetraosylceramide was identical with that of a ganglioside $G_{D1\alpha}$ isolated from rat ascites hepatoma AH7974F cells, the ceramide composition of the former clearly differed from that of the latter. A similar distribution of these gangliosides was seen among hemisph, diencephalon, and a mixed tissue of opticlobe, cerebellum, and medulla oblongata of the brain. The brain tissues of *Rana nigromaculata* and *Rhacophorus arboreus* also included the same gangliosides as those of bull frog.

Many types of ganglioside have been found in brain and in other nervous system tissue and also on cell surfaces in mammals, birds, reptiles, and fish. Several of them are receptors for toxins and hormones[1,2] and also antigens of specific monoclonal antibodies.[3,4] Recently, four gangliosides were isolated from bull frog (*Rana catesbeiana* Shaw) brain and the partial structures of their carbohydrate parts were proposed on the basis of detection of a monosialosyl ganglioside and asialo-G_{M1} on t.l.c. of a hydrolysate obtained from the gangliosides by treatment with neuraminidase.[5,6] Reference 5 describes the t.l.c. of the monosialosyl ganglioside which shows a spot between G_{M1} and G_{D1a}, but reference 6 reports this ganglioside as showing the same mobility on t.l.c. as that of G_{M1}. On the basis of these t.l.c. data, both the references propose that the gangliosides isolated from the frog brain have structures involving a linkage of *N*-acetylneuraminyl-(2→6)-*O*-*N*-acetyl-D-galactosamine.

We have established the structures of three major gangliosides present in the brain of bull frog by a combination of ^{1}H n.m.r. and mass spectral measurements and chemical methods and their distributions on hemisph, diencephalon, and mixed tissue of opticlobe, cerebellum, and medulla oblongata of the brain of *Rana catesbeiana* and in the brains of *Rana nigromaculata* Hallowell and *Rhacophorus arboreus* (Y. Okada et Kawano).

Results and Discussion

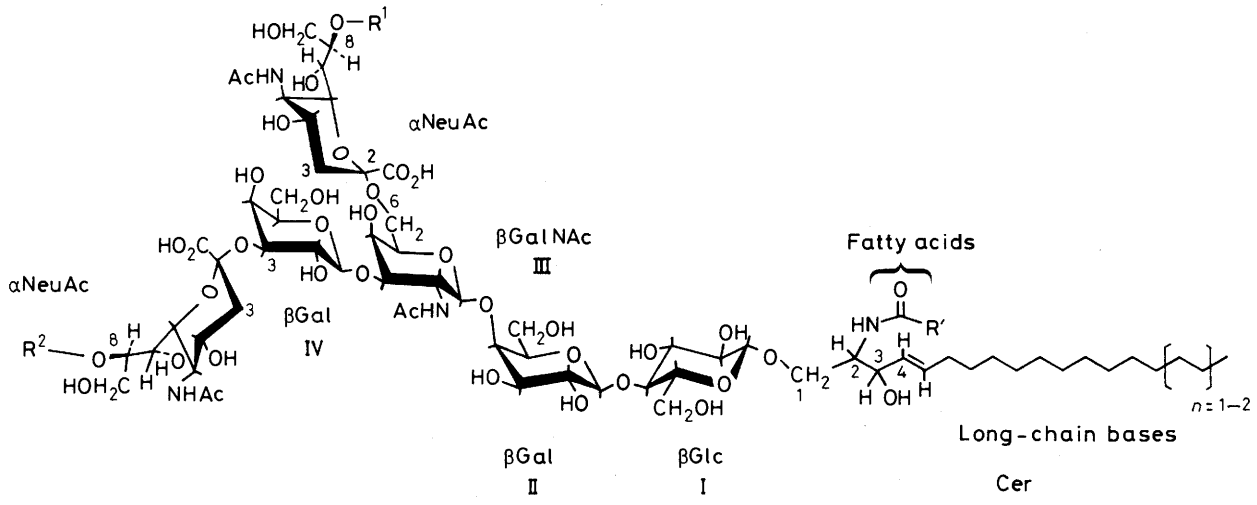
Isolation of Gangliosides.—Fresh bull frog (*R. catesbeiana*) brain tissue, after removal of fats by treatment with acetone, was extracted with a mixture of chloroform and methanol followed by hot ethanol to give crude glycolipids. These glycolipids were then separated into neutral and acidic glycolipids. To remove alkaline labile contaminants, the acidic glycolipids were hydrolysed with 0.5 mol dm^{-3} NaOH under mild conditions to give a mixture of gangliosides. T.l.c. of the mixture showed the presence of three major and two minor gangliosides. Mobilities of these gangliosides on t.l.c. with two solvent systems (A and B) differed from those of a series of authentic gangliosides present in bovine brain, as given in Table 1. Repeated column chromatography of the mixture of gangliosides gave three gangliosides, (**1**), (**2**), and (**3**), as the major constituents. These gangliosides gave a single band on t.l.c. developed with two solvent systems.

Structures of the Carbohydrate Part of the Gangliosides.—The structures of the three gangliosides were established as (**1**), (**2**), and (**3**) mainly by ^{1}H n.m.r. and mass spectral measurement. The gangliosides are composed of an oligosaccharide core which comprises an oligosaccharide residue and *N*-acetylneuraminic acid and a ceramide part which comprises fatty acids and long-chain bases. The structures of the carbohydrate part of the

Table 1. Compositions of the gangliosides[a] in frog and bovine brain

	Relative content (%)								
	(**3**)	(**2**)	Uc[b]	G_{T1b}	G_{D1b}	(**1**)	G_{D1a}	U_C	G_{M1}
Brain	(0.05)[c]	(0.10)	(0.13)	(0.18)	(0.21)	(0.26)	(0.28)	(0.29)	(0.35)
Frog	25.9	16.7	Tr.[d]	N.d.[e]	N.d.	57.4	N.d.	Tr.	N.d.
Bovine	N.d.	N.d.	N.d.	6.7	2.6	N.d.	33.1	N.d.	47.6

[a] Gangliosides of bovine brain are abbreviated according to Svennerholm (1964) and the nomenclature designated according to the recommendation of the IUPAC Nomenclature Committee (IUPAC, 1977). G_{M1}: II^{3}NeuAc-GgOse$_4$Cer, G_{D1a}: II^{3}NeuAcIV^{3}NeuAc-GgOse$_4$Cer, G_{D1b}: II^3(NeuAc)$_2$-GgOse$_4$Cer, G_{T1b}: II^3(NeuAc)$_2$IV^{3}NeuAc-GgOse$_4$Cer. [b] Unidentified constituent. [c] R_F values with the solvent system A were as shown in parentheses. [d] Trace amount (<0.1%). [e] Not detected.



(1) $R^1 = R^2 = H$

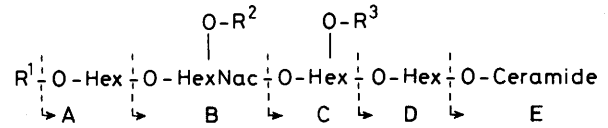
(2) $R^1 = H$, $R^2 = NeuAc$

(3) $R^1 = R^2 = NeuAc$

Table 2. Molecular and fragment ions in the f.a.b.-mass spectra of gangliosides (**1**)—(**3**)

	Mass number (*m*/*z*)					
Ganglioside	(*M* + Na − H)	A	B	C	D	E
(**1**)	1 858	1 544	1 382	888	726	565
					698	536
(**2**)	2 149	1 544	1 382	888	726	564
					698	536
(**3**)	n.d.[a]	n.d	n.d.	888	726	564
G_{D1a}[b]	1 886[c]	1 572	1 410	1 207	754	592
	1 858[c]	1 544	1 382	1 179	726	564

Proposed fragmentation pattern



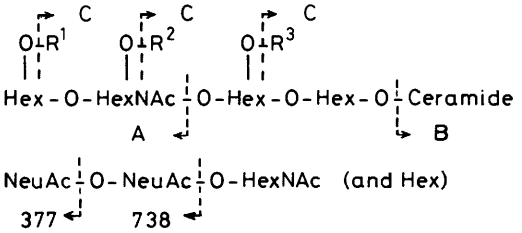
G_{D1a}: R^1 = NeuAc, R^2 = H, R^3 = NeuAc

[a] Not detected. [b] All the ions were assigned on the basis of the reported spectral data.[7] [c] The difference of 28 in the mass number was due to the difference in chain length of the long-chain bases in the ceramide component.[7]

Table 3. Molecular and fragment ions in the s.i.-mass spectra of the permethylated derivatives of (**1**)—(**3**)

	Mass number (*m*/*z*)				
Ganglioside	(*M* + Na − H)	(*M* + H)	(A + H)	(B + H)	(C + H)
(**1**)	2 210	2 187[a]	1 187	577	377
		2 159[a]		549	
(**2**)	2 571	2 548	1 548	577	738, 377
		2 520		549	
(**3**)	2 932	2 909	1 909	577	738, 377
		2 881		549	
G_{D1a}	2 238	2 215	826	604	377
	2 210	2 187		577	
				549	

Proposed fragmentation pattern



[a] The difference of 28 in the mass number was probably due to the difference in chain length of the long-chain bases or fatty acids in the ceramide component.

gangliosides were elucidated on the basis of the ^1H n.m.r. and mass spectral and chemical evidence, as follows.

(*a*) *Ganglioside* (**1**). This compound showed a molecular ion at *m*/*z* 1 858 (*M* + Na − H) in the f.a.b.-mass spectrum. This indicates that the ganglioside is a disialosyltetrahexosylceramide including two molecules of *N*-acetylneuraminic acid (NeuAc). Table 2 gives the f.a.b.-mass spectra of three gangliosides, (**1**)—(**3**). A series of fragment ions at *m*/*z* 1 544 (A), 1 382 (B), 726 (D), and 564 (E) in the f.a.b.-mass spectrum of (**1**) coincided with the corresponding ions of an authentic G_{D1a}.[7] This indicates that a partial structure of (**1**) is hexosyl-*O*-*N*-acetylhexosaminyl-*O*-hexosyl-*O*-hexoside of a ceramide (Hex-*O*-HexNAc-*O*-Hex-*O*-Hex-*O*-Cer), in which one molecule of NeuAc binds to Hex or HexNAc. A fragment ion at *m*/*z* 888 (C) appeared characteristically in the f.a.b.-mass spectra of three gangliosides, (**1**)—(**3**), indicating the presence of a partial structure, Hex-*O*-Hex-*O*-Cer, freed of NeuAc. These fragment ions in the mass spectra indicate that both the HexNAc and terminal Hex in the carbohydrate part of (**1**) is bound with one molecule of NeuAc. Table 3 gives the s.i.-mass spectra of the permethylated derivatives of (**1**)—(**3**). The linkages between NeuAc and HexNAc and another NeuAc and terminal Hex were supported by two fragment ions at *m*/*z* 1 187 (A + H) and 377 (C + H) in the s.i.-mass spectrum of the permethylated derivative of (**1**). In the f.a.b.-mass spectra of (**1**) and (**2**), two fragment ions, (D) and (E), due to Hex-*O*-Cer and Cer appeared as a set of ions having a difference of 28 in the mass number (Table 2). This difference in the mass number was similarly observed for the molecular ions (*M* + H) and fragment ions (B + H) in the s.i.-mass spectra of the three permethylated

Table 4. ^{1}H N.m.r. chemical shifts of gangliosides (**1**)—(**3**). Coupling constants are given in parentheses

	Methyl and methylene protons[a]				
	NeuAc (COMe)	GalNAc (COMe)	Cer[b] (Me)	Cer (CH_2)	NeuAc[c] (3-H_e, -H_a)[d]
(**1**)	1.89	1.82	0.86	1.23	2.62 (H_e), 1.35 (H_a); 2.71 (H_e), 1.23 (H_a)
	1.90				(5.0, 12.0), (12.0, 12.0); (11.5, 4.8)
(**2**)	1.87	1.83	0.85	1.23	2.63 (H_e), 1.35 (H_a); 2.74 (H_e), 1.23 (H_a)
	1.88				(5.0, 12.0), (12.0, 12.0); (11.5, 4.8)
(**3**)	1.87	1.80	0.86	1.23	2.64 (H_e), 1.35 (H_a); 2.76 (H_e), 1.23 (H_a)
	1.89				(5.0, 12.0), (12.0, 12.0); (11.5, 4.8)

	Anomeric protons[a]				Olefinic protons[a,e]		Amido protons[a]		
	βGlc I	βGal II	βGalNAc III	βGal IV	4-H	5-H	Cer	GalNAc	NeuAc
(**1**)	4.15	4.19	4.51	4.23	5.33	5.54	7.47	7.47	8.09, 8.15
	(6.1)	(7.3)	(7.4)	(6.7)	(7.2, 14.9)	(7.0, 14.9)	(8.0)	(8.0)	(8.0), (8.0)
	4.18	4.19	4.56	4.21[f]	5.31	5.50[f]	—	—	—[f]
	(6.1)	(7.3)	(7.4)	(6.7)	(7.2, 14.9)	(7.0, 14.9)			
(**2**)	4.16	4.20	4.53	4.23	5.32	5.50	7.45	7.45	8.03, 8.09
	(6.1)	(7.3)	(7.4)	(7.3)	(7.0, 15.0)	(8.0, 15.0)	(9.2)	(9.2)	(7.6), (7.6)
(**3**)	4.16	4.20	4.52	4.22	5.31	5.50	7.46	7.46	8.02, 8.02
	(6.8)	(6.4)	(7.3)	(6.8)	(6.4, 14.9)	(7.0, 14.9)	(8.4)	(8.4)	(br d), (br d)

Abbreviations; βGal: β-D-galactopyranose, βGalNAc: 2-acetylamino-2-deoxy-β-D-galactopyranose, βGlc: β-D-glucopyranose, Cer: ceramide. [a] Obtained in $(CD_3)_2SO$ at 30 °C. [b] Proton signal for methyl group of fatty acids overlapped with that of the methyl group of long-chain bases. [c] Proton signals for equatorial 3-H of the extra NeuAc residues in (**2**) and (**3**) probably overlapped with a strong signal due to the solvent at δ 2.50. [d] The proton signals at δ 2.62 and 2.71 showed a cross peak to the proton signals at δ 1.35 and 1.23, respectively, in the homonuclear ^{1}H-^{1}H shift correlation spectrum of (**1**) which was obtained in $(CD_3)_2SO$ at 30 °C. [e] Olefinic proton signals due to fatty acids appeared at δ 5.30—5.31 as a weak triplet (*J* 4.4—4.5). [f] Obtained in $(CD_3)_2SO$–D_2O (2:1, v/v) at 90 °C.

derivatives (Table 3). These findings indicate that the ceramide parts of (**1**)—(**3**) have a variation in their chain length.

The structure of the carbohydrate part of (**1**) was elucidated on the basis of the ^{1}H n.m.r. spectrum, as follows. In the ^{1}H n.m.r. spectra taken in $(CD_3)_2SO$ at 30 °C and $(CD_3)_2SO$–D_2O at 90 °C, the signals due to the methyl and methylene protons, four anomeric protons, olefinic protons, and amido protons of (**1**)—(**3**) were assigned as given in Table 4 by comparing their chemical shifts and coupling constants with those of the corresponding protons of authentic glycosphingolipids[8–10] and $G_{D1\alpha}$ isolated from rat ascites hepatoma AH7974F cells.[11] In the ^{1}H n.m.r. spectra of (**1**)—(**3**) in $(CD_3)_2SO$ solution at 30 °C, two double doublets at δ 2.62—2.64 and 2.71—2.76 were assigned to equatorial 3-H protons of αNeuAc linked to the 6-position of 2-(acetylamino)-2-deoxy-D-galactopyranose (GalNAc) and of αNeuAc linked to the 3-position of a terminal D-galactopyranose (Gal), respectively, by coincidence of their chemical shifts and coupling constants with those of the corresponding protons of $G_{D1\alpha}$.[11] Further, the chemical shifts and coupling constants of the four anomeric protons of (**1**) in the ^{1}H n.m.r. spectrum taken in $(CD_3)_2SO$–D_2O solution at 90 °C coincided with those of the corresponding protons of $G_{D1\alpha}$. These findings indicate that (**1**) has the same carbohydrate entity as that of $G_{D1\alpha}$.

This structure of the carbohydrate of (**1**) was supported by the formation of 1,3,5-tri-*O*-acetyl-2,4,6,-tri-*O*-methylgalactitol (2,4,6-tri-*O*-MeGal), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (2,3,6-tri-*O*-MeGal), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol (2,3,6-tri-*O*-MeGlc), and 1,3,5,6-tetra-*O*-acetyl-4-mono-*O*-methyl-2-*N*-methylacetamide-2-deoxygalactosaminitol (4-mono-*O*-MeGalNAcMe) from the permethylated derivative of (**1**) by hydrolysis, reduction, and acetylation, successively. Thus, the structure of (**1**), except for the ceramide part, was found to be disialosylgangliotetraosylceramide (III^6NeuAcIV^3NeuAc-GgOse$_4$Cer).

(*b*) *Ganglioside* (**2**). This compound was characterized as a trisialosyltetrahexosylceramide including three molecules of NeuAc by appearance of the molecular ions in the f.a.b.-mass spectrum of (**2**) itself and in the s.i.-mass spectrum of the permethylated derivative of (**2**), as given in Tables 2 and 3, respectively. That the oligosaccharide sequence in the carbohydrate part of (**2**) was the same as that of (**1**) was established by coincidence of the f.a.b.-mass fragmentation patterns (Table 2). Furthermore, a partial structure, NeuAc-*O*-NeuAc-*O*-Hex-*O*-HexNAc-*O*-NeuAc, was suggested by the appearance of three fragment ions at *m/z* 1 548 (A + H), 738 (C + H), and 377 (C + H) in the s.i.-mass spectrum of the permethylated derivative (Table 3). The ^{1}H n.m.r. spectrum of (**2**) in $(CD_3)_2SO$ solution at 30 °C coincided with the corresponding spectrum of (**1**) (Table 4). This indicates that the carbohydrate part of (**2**) has the same structure as that of (**1**). The structure of the carbohydrate part was further supported by the formation of 2,4,6-tri-*O*-MeGal, 2,3,6-tri-*O*-MeGal, 2,3,6-tri-*O*-MeGlc, and 4-mono-*O*-MeGalNAcMe, from the permethylated derivative in the same manner as for (**1**).

The position of linkage between NeuAc and another NeuAc linked to the 3-position of terminal βGal was not determined by the spectral and chemical evidence. However, NeuAc-*O*-NeuAc included in the naturally occurring gangliosides is known to have only an α(2→8) linkage.[12] The presence of this type of linkage in the carbohydrate part of (**2**) is supported by the fact that a monosialosyl ganglioside is known to be given from the gangliosides isolated from the bull frog brain by hydrolysis of αNeuAc-(2→8)-*O*-NeuAc with neuraminidase.[5,6] Thus, the structure of ganglioside (**2**) was shown to be trisialosylgangliotetraosylceramide [III^6NeuAcIV^3(NeuAc)$_2$-GgOse$_4$Cer].

(*c*) *Ganglioside* (**3**). This compound was characterized as a tetrasialosyltetrahexosylceramide including four molecules of NeuAc and a partial structure, Hex-*O*-Hex-*O*-Cer, on the basis of two fragment ions (D and E) in the f.a.b.-mass spectrum of (**3**) and the molecular ions in the s.i.-mass spectrum of its permethylated derivative, as given in Tables 2 and 3, respect-

Table 5. Ceramide compositions of gangliosides (**1**), (**2**), and (**3**)

	(1)	(2)	(3)
Fatty acids (%)			
C_{14}:0	7.6	2.7	12.4
C_{15}:0	4.6	2.5	6.0
C_{16}:1	2.7	5.8	Tr.[a]
C_{16}:0	43.0	36.0	61.2
C_{17}:0	2.3	2.6	1.8
C_{18}:1	8.4	19.5	Tr.
C_{18}:0	24.1	31.7	15.6
C_{20}:0	1.2	Tr.	Tr.
C_{22}:0	6.1	Tr.	3.3
Long-chain bases (%)			
d_{18}:1[b]	55.7	78.4	54.1
d_{20}:1	44.2	21.6	45.9

[a] Trace amount (<0.1%). [b] The symbolism d_{18}:1, for example, indicates a dihydroxy C_{18} long-chain base with one double bond following the established usage for long-chain base derivatives.

Table 6. Compositions of the gangliosides in bull frog brain tissue and its constituent parts

	Relative content (%)				
Tissue	Uc[a]	(1)	(2)	Uc	(3)
Total	Tr.[b]	57.4	16.7	Tr.	25.9
Hemisph	Tr.	55.0	28.9	Tr.	15.1
Diencephalon	Tr.	47.8	38.3	Tr.	13.9
Opticlobe, Cerebellum, and Medulla oblongata	Tr.	46.7	29.7	Tr.	23.6

[a] Unidentified constituent. [b] Trace amount (<0.1%).

ively. A partial structure, NeuAc-*O*-NeuAc-*O*-Hex-*O*-HexNAc-*O*-NeuAc-*O*-NeuAc, was indicated by appearance of a series of fragment ions (A + H and C + H) in the s.i.-mass spectrum of the permethylated derivative (Table 3). Confirmation of the same structure as that of (**1**) and (**2**) in the carbohydrate part of (**3**) was determined by coincidence of the ^{1}H n.m.r. spectrum with the spectra of (**1**) and (**2**) (Table 4). This structure of the carbohydrate part was further supported by the formation of the same partially methylated alditol acetates as those of (**1**) and (**2**). Thus, the structure of the ganglioside (**3**) was shown to be tetrasialosylgangliotetraosylceramide [III^6(NeuAc)$_2$IV^3-(NeuAc)$_2$-GgOse$_4$Cer].

Ceramide Compositions of Gangliosides.—Three permethylated derivatives of (**1**)—(**3**) were hydrolysed with 1.4 mol dm^{-3} H_2SO_4–30% acetic acid to give fatty acid and 3-mono-*O*-methyl-2-*N*-methyl long-chain bases. The fatty acids and long-chain bases were methylated and trimethylsilylated, respectively, to afford their methyl esters and TMS-derivatives. Table 5 gives compositions of these components. The fatty acids of the three gangliosides were mainly composed of hexadecanoic acid C_{16}:0 (36—61%) and octadecanoic acid C_{18}:0 (24—32%). A predominant fatty acid of (**2**) was octadecenoic acid C_{18}:1 (*ca.* 20%). The long-chain bases were mainly composed of 2-amino-1,3-dihydroxyoctadec-4-ene (sphingosine, d_{18}:1) and 2-amino-1,3-dihydroxyicos-4-ene (icosasphingosine, d_{20}:1). Although (**1**) had the same carbohydrate part as that of $G_{D1\alpha}$, its ceramide composition clearly differed from that of $G_{D1\alpha}$ which has docosanoic acid C_{22}:0 (27%) and tetracosanoic acid C_{24}:0 (21%) as predominant fatty acids and 1,3-dihydroxy-2-aminooctadecane (dihydrosphingosine, d_{18}:0) (51%) as a predominant long-chain base.[11] The long-chain bases of (**1**)—(**3**) were found to have a double bond at the 4-position on the basis of two olefinic proton signals at δ 5.31—5.33 (4-H) and 5.50—5.54 (5-H) in the ^{1}H n.m.r. spectra (Table 4). The large coupling constants ($J_{4,5}$ 14.9—15.0) between the 4-H and 5-H indicate that this double bond is *trans*. The signals for 2-H and 3-H were difficult to observe due to overlap with methine signals of oligosaccharide residue and NeuAc. Thus, the ceramide parts of the three gangliosides, (**1**)—(**3**), were found to be mainly composed of hexadecanoic acid and octadecanoic acid and sphingosine and icosasphingosine.

Distribution of Gangliosides in the Brain.—The brain tissue of bull frog was divided into hemisph, diencephalon, and mixed opticlobe, cerebellum, and medulla oblongata tissue, due to the difficulty in separation of these from each other. Table 6 gives compositions of the gangliosides present in these three parts. The same gangliosides as those in the total brain tissues were uniformly present in the three parts and these parts showed a similarity in the composition.

T.l.c. of the brain gangliosides of *R. nigromaculata and Rh. arboreus* showed the presence of three major and two minor gangliosides having the same mobilities as those of the corresponding gangliosides present in the brain of bull frog (Table 1). The gangliosides of the two frogs, on column chromatography, gave major gangliosides having the same mobility on t.l.c. as that of (**1**) of bull frog. The s.i.-mass spectra of their permethylated derivatives coincided with the spectrum of the permethylated derivatives of (**1**). Thus, the gangliosides isolated from the brain of bull frog were commonly present in the brains of other frogs.

The structures of the major gangliosides present in the brain of frogs were thus established on the basis of the ^{1}H n.m.r. and mass spectral and chemical evidence. The frog gangliosides had a common skeletal structure, gangliotetraosylceramide, which is widely involved in the gangliosides present in brain and nervous systems of other animal species. However, the frog gangliosides had a unique linkage of αNeuAc-(2→6)-*O*-GalNAc which has not been seen in gangliosides of other animal species. This linkage is known to be involved in glycoproteins including *O*-linked oligosaccharides in mucin-type glycoprotein[13] and a disialosylgangliotetraosylceramide $G_{D1\alpha}$ of rat ascites hepatoma AH7974F cells.[11]

Experimental

T.l.c. was performed on pre-coated silica gel plates (Merck, GF_{254}, 0.25 mm). Gangliosides were visualized by spraying the plates with resorcinol–HCl reagent. The following solvent systems were used for t.l.c.: A, chloroform–methanol–water containing 0.2% $CaCl_2$ (60:40:9, v/v/v); B, chloroform–methanol–0.25 mol dm^{-3} ammonia (60:35:8). Bands of gangliosides were analysed with a densitometer and visualized with light λ = 580 nm. For detecting permethylated gangliosides, the plates developed with chloroform–methanol (95:5) were sprayed with anthrone–H_2SO_4 reagent. G.l.c. was performed on Shimadzu GC-15A equipped with f.i.d. and 3 m × 2.6 mm i.d. glass column packed with one of 2% OV-101, 2% OV-17, and 1.5% OV-225 on chromosorb W-AWDMCS (80—100 mesh). F.a.b.-mass spectra were obtained by negative ion detection with a JEOL JMS HX-110 equipped with a xenon beam generating system as a f.a.b.-ion source and a JMA-5000 computer system. Analytical conditions were as follows: a sample of ganglioside (5 μg) dissolved in chloroform–methanol (1:1; 2 μl); matrix, mixture of triethanolamine (2 μl), and 1,1,3,3-tetramethylurea (1 μl); accelerating voltage, 10 keV. Assignment of mass number was achieved by comparing the spectra with the mass spectrum of CsI. S.i.-mass spectra were obtained by detecting positive ions with a Hitachi M-80B double focussing mass spectrometer

equipped with a M-8086 xenon beam generating system and a M-0101 data processing system. Analytical conditions were as follows: a sample of permethylated ganglioside (10 μg) dissolved in chloroform–methanol (2:1; 1 μl); matrix, mixture of triethanolamine (2 μl) and 0.1% $MeCO_2Na$ in methanol (1 μl); accelerating voltage, 1.5 keV. Assignment of mass number was achieved by comparing the spectra with the mass spectrum of Fomblin which is the mass standard substance sold by Gaskura Kogya Inc. and has the formula $[(OCFCF_3CF_2)_n(OCF_2)_m]$. G.l.c.–m.s. was performed on a Shimadzu QP-1000 equipped with above columns. E.i.-mass spectra were obtained at 70 eV and 200 °C of ion source temperature. ^1H N.m.r. spectra were recorded on a JEOL GX-400 spectrometer in $(CD_3)_2SO$ at 30 °C and $(CD_3)_2SO$–D_2O (2:1) at 90 °C with Me_4Si as internal standard.

Isolation of Gangliosides.—Fresh bull frog brains (904 specimens, 337 g) were ground in a mortar after freezing with liquid N_2. The ground brain tissue was treated with acetone (3 000 cm^3) to remove fats. After filtration, the insoluble residue (42 g) was extracted with chloroform–methanol (1:1; 3 000 cm^3) followed with hot ethanol (60 °C; 1 000 cm^3). The combined extracts, after removal of solvent under reduced pressure, were dialysed against distilled water for 1 day at 5 °C. The content was then lyophilized to give a mass of crude glycolipids (2.643 g). The crude glycolipids were subjected to chromatography on DEAE-Sephadex A-25 (acetate form). Elution with chloroform–methanol–water (3:7:1; 600 cm^3) gave an eluate containing neutral glycolipids. Elution with chloroform–methanol–0.8 mol dm^{-3} ammonium acetate (3:7:1; 1760 cm^3) gave an eluate containing acidic glycolipids. This eluate was concentrated to 100 cm^3 under reduced pressure and the concentrate was then dialysed against distilled water. The content was lyophilized to give acidic glycolipids (626 mg). To remove alkaline labile contaminants, the acidic glycolipids were hydrolysed with methanolic 0.5 mol dm^{-3} NaOH (300 cm^3) at 40 °C for 1 h and then neutralized with acetic acid. The neutral solution was concentrated to 100 cm^3 under reduced pressure and the concentrate was then dialysed against distilled water. The content was lyophilized to give a mixture of gangliosides (600 mg) which was then subject to chromatography on Iatrobeads (Iatron, Tokyo) (4 g). Fraction 1 (500 mg) mainly containing ganglioside (**1**) was first eluted with 300 cm^3 of chloroform–methanol–water (75:22:3, solvent C). Fraction 2 (39 mg) mainly containing two gangliosides, (**2**) and (**3**), was next eluted with 800 cm^3 of chloroform–methanol–water (61:36:3, solvent D). Fraction 3 (31 mg) mainly containing ganglioside (**3**) was finally eluted with 800 cm^3 of chloroform–methanol–water (41:56:3, solvent E). Fraction 1 was re-chromatographed on Iatrobeads (2 g) with solvent C to give ganglioside (**1**) (33 mg) and an unidentified constituent (2.2 mg). Fracton 2 was re-chromatographed on the same adsorbent with solvent D to give ganglioside (**2**) (26.4 mg), ganglioside (**3**) (4.8 mg), and another unidentified constituent (0.6 mg). Fraction 3 was also re-chromatographed on the same adsorbent with solvent E to give ganglioside (**3**) (5.3 mg). Purity of the gangliosides was checked by t.l.c. with two solvent systems (A and B).

Methylation of the Three Gangliosides and G_{D1a}.—A typical procedure was as described below. Following the method of Hakomori,[14] ganglioside (*ca.* 1 mg) was permethylated with methyl iodide and sodium hydride in Me_2SO. The crude permethylated derivative was applied to Sep-Pak cartridge (silica, Waters) to remove a large excess of reagent by elution with chloroform (10 cm^3), and the permethylated derivative was eluted with chloroform-methanol (1:1; 10 cm^3) from this cartridge. The purity of the permethylated derivative was checked with t.l.c. developed with chloroform–methanol (95:5).

Preparation of Methyl Esters of Fatty Acids, TMS-Derivatives of Partially Methylated Long-Chain Bases, and Partially Methylated Alditol Acetates.—Following the reported method,[15] the permethylated derivative (*ca.* 1 mg) dissolved in a mixture (1 cm^3) of 1.4 mol dm^{-3} H_2SO_4–80% acetic acid (1:1) was heated at 80 °C for 20 h. The reaction mixture was extracted with diethyl ether (10 cm^3 × 3) to give fatty acids. The fatty acids were methylated with diazomethane. T.l.c. of the resulting methyl esters with light petroleum (b.p. 30—60 °C)–diethyl ether (7:3) showed the presence of non-hydroxy fatty acids (R_F, 0.8) and trace amount of hydroxy fatty acids (R_F, 0.1).

The reaction mixture, after removal of the fatty acids, was treated with Dowex-WGR. The neutralized solution obtained was extracted with diethyl ether (10 cm^3 × 3) to give partially methylated long-chain bases. The partially methylated long-chain bases were trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane in pyridine, following the reported method.[16]

To the residual aqueous solution free from partially methylated long-chain bases, 0.7 cm^3 of 1% $NaBH_4$ was added and, after 4 h, any excess of borohydride was quenched by adding a few drops of glacial acetic acid. The reaction solution was, after evaporation to dryness, treated with 0.7 cm^3 of acetic anhydride–pyridine (1:1) at 80 °C for 14 h. The reaction mixture was partitioned with chloroform–methanol–water (1:1:0.9) to give partially methylated alditol acetates in the lower organic phase.

Analysis of the Methyl Esters of Fatty Acids, TMS-Derivatives of Partially Methylated Long-chain Bases, and Partially Methylated Alditol Acetates.—G.l.c. of the methylated fatty acids and TMS-derivatives of partially methylated long-chain bases were performed under the following conditions: columns, 2% OV-101 and 2% OV-17; injection temp., 280 °C; column temp., 150—250 °C at 2 °C min^{-1}. Partially methylated alditol acetates were analysed under the following g.l.c. conditions: column, 1.5% OV-225; injection temp., 220 °C; column temp., 170—200 °C at 2 °C min^{-1}. G.l.c.–m.s. was performed under the same conditions as above. Composition (%) of the product was obtained on the basis of the peak area in g.l.c.

The methyl esters of fatty acids were identified by the coincidence of the e.i.-mass fragmentation patterns with those of authentic materials and the reported spectral data.[17] The TMS-derivatives of the partially methylated long-chain bases were identified by the coincidence of the e.i.-mass fragmentation patterns with those of the corresponding derivatives obtained from the permethylated derivative of G_{D1a}. The partially methylated alditol acetates were identified by the coincidence between their e.i.-mass fragmentation patterns and the reported spectral data.[11,18] Table 5 gives the compositions of the methyl esters of fatty acids and TMS-derivatives of partially methylated long-chain bases. Table 7 gives structures of four partially methylated alditol acetates.

Distribution of Gangliosides in the Brain.—The bull frog brain tissue was divided into hemisph (18 g), diencephalon (5 g), and mixed (38 g) opticlobe, cerebellum, and medulla oblongata tissue. Following the procedure for the total brain tissue, the three parts were treated to give a mixture of gangliosides, 80, 59, and 81 mg, respectively. Compositions of gangliosides are given in Table 6.

Following the procedure as described above, the *Rana nigromaculata* (110 specimens) brain tissue (7 g) was treated to give a mixture of gangliosides (50 mg). Repeated column chromatography of the mixture of gangliosides on Iatrobeads with three solvent systems (C, D, and E) gave a major (1.0 mg) and two minor (trace amount) gangliosides. Almost the same amount of a major and two minor gangliosides were also isolated from

Table 7. Structures of partially methylated alditol acetates

Gangliosides	2,4,6-tri-*O*-Me-Gal	2,3,6-tri-*O*-Me-Gal	2,3-di-*O*-Me-Gal	2,3,6-tri-*O*-Me-Glc	4-mono-*O*-Me-GalNAcMe	4,6-di-*O*-Me-GalNAcMe
(1)	+	+	−	+	+	−
(2)	+	+	−	+	+	−
(3)	+	+	−	+	+	−
G_{D1a}	+	−	+	+	−	+

+ and − denote yielded and not yielded, respectively.

Rhacophorus arboreus (103 specimens) brain tissue (10 g) in the same manner as above. The mobilities of these gangliosides on t.l.c. with two solvent systems (A and B) were identical with those of three ganglioside, (**1**)–(**3**). Each of the major gangliosides having the same mobility as that of (**1**) was then methylated by the Hakomori method [13] to give their permethylated derivatives. The s.i.-mass spectra of the permethylated derivatives coincided with the spectrum of the permethylated derivative of (**1**).

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