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Naturally occurring ganglioside lactones in Minke whale brain

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Abstract

Ganglioside lactones in Minke whale brain were analysed. In order to exclude the possibility of the artificial lactonization of gangliosides during the analytical procedures, lactone species were separated from their parent gangliosides at an early stage by DEAE-Sephadex A-25 column chromatography using a stepwise elution. Applying the improved method, GM4[1] lactones were found in gray and white matters of cerebrum and cerebellum, while GD3 monolactone was found only in cerebellar gray matter. The concentration of each ganglioside lactone was one thousandth of the parent ganglioside.

Keywords: Gangliosides; Naturally occurring ganglioside lactones; Brain tissues

1. Introduction

The presence of ganglioside lactones was first postulated by Kuhn and Muldner in 1964 [1] and by Wiegandt in 1966 [2]. Since this time, attention has been focused on the natural occurrence, chemical structures and biological functions of ganglioside lactones. By proton NMR spectroscopy, it has been shown that ganglioside lactones include NeuAcl-9NeuAc and/or NeuAcl-2Gal linkages [3–8]. Recently, we found a new type of linkage, NeuAcl-4Gal, in GM4 lactone (GM4LM) which was isolated from the whole brain of bryde's whale (*Baraenoptera edeni*) [9]. Each type of lactone formed by the three different linkages was found to display an identical negative circular dichroism (CD) band at 235 nm [10]. Using the CD band, we developed another method to estimate lactones not only in gangliosides [11] but also in oligo-/polymers of sialic acid such as a colominic acid [12]. However, because of the lability of lactones and their occurrence in only trace amounts in tissues, there has been controversy whether the lactones are the original components or artificial ones formed during the isolating processes. Gross et al. [13] and Nores et al. [14] treated native cells or

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[1] Abbreviations: gangliosides are named according to the Svennerholm nomenclature [29] and the IUPAC-IUB recommendations [30]. GM4LMs, two GM4-lactones containing NeuAcl-2Gal and NeuAcl-4Gal lactone linkage, respectively; GD3LM, GD3-monolactone containing NeuAcl-9NeuAc lactone linkage; GDlbLM, GDlb monolactone containing NeuAcl-9NeuAc lactone linkage; GM4Me, methyl ester of GM4; GD3Me, methyl ester of GD3; GDlbMe, methyl ester of GDlb. PS, phosphatidylserine. PI, phosphatidylinositol. PC, phosphatidylcholine. PE, phosphatidylethanolamine.

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tissue homogenate directly with ^{3}H labelled borohydride and detected ^{3}H labelled ganglioside-ols. By this method, not only lactone species but also esters of sialic acid carboxyl groups were reduced to the same ^{3}H labelled derivatives. Riboni et al. [15] reported that, after the intracisternal injection of ^{3}H labelled GDlb, ^{3}H labelled GDlb lactone was detected in rat brain extract. More recently, Bouchon et al. [16] produced a monoclonal antibody (BBH5) directed to the lactone ring formed by NeuAcl-9NeuAc linkage and detected GDlb lactone and GD3 lactone in freshly prepared ganglioside fractions from human and mouse brains by TLC immunostaining. In these studies, however, preparations of ganglioside lactones were performed in the presence of their parent gangliosides and therefore the possibility of the artificial formation of lactone species could not be excluded.

In the present study, a conventional analytical method for brain gangliosides was improved to separate lactone species from their parent gangliosides at an early stage in the process and to prevent hydrolysis of lactone linkage for higher recovery. By the improved method, ganglioside lactones in brain tissues of Minke whale were analysed.

2. Results and discussion

Brain gangliosides are usually analyzed by the combination of two types of column chromatography using DEAE-Sephadex A-25 and Iatrobeads [17]. By DEAE-Sephadex A-25 column chromatography, the total lipid extract is divided into two fractions; neutral lipids which pass through the column and acidic lipids, including gangliosides, which are retained on the column. Three problems arise when ganglioside lactones are analyzed by the conventional method. (i) As lactones always coexist with their parent gangliosides throughout the analysis, a possibility of artificial lactone formation during the analytical procedures cannot be excluded. (ii) Because of omission of the mild alkali treatment, acidic phospholipids (e.g. PS and PI) remain in the ganglioside fraction. Due to the extra amount of coexisting lipids, the amount of ganglioside sample that can be applied on a two-dimensional TLC plate is limited to about 5 μg as total ganglioside bound sialic acid. Lactone components which are less than 1% of total ganglioside sialic acid are under the limit of detection by the resorcinol method. (iii) The recovery is too low to determine the labile lactones which are contained in very small amount in the brain tissues. Therefore, the conventional method was improved to separated lactones from their parent gangliosides at the early step of the analysis and to prevent the hydrolysis of lactone linkage.

Lactones in the acidic lipid fraction.—As shown in Fig. 1A, GDlbLM and GDlb in the starting sample were eluted into monosialoganglioside and disialoganglioside fractions, respectively, from a DEAE-Sephadex A-25 column by a stepwise elution. In the monosialoganglioside fraction, GDlbLM and another resorcinol-positive band of GDlbMe, which was not present in the starting sample, were detected. Recovery of GDlbLM at each step, estimated by both CD spectrometry and TLC-densitometry is shown in Table 1. The data obtained by the two methods agreed well with each other. The measurement of ellipticity at 235 nm enabled us to determine the ganglioside lactone content, even in the presence of other components such as the parent gangliosides and/or their methyl esters without the concentration of the fraction. As shown in Fig. 1B, after desalting by gel filtration, GDlb was not present in a detectable amount in the GDlbLM fraction and overall recovery of GDlbLM was 60%. On the other hand, desalting by dialysis made the recovery of GDlbLM down to about 30%, due to the partial hydrolysis of GDlbLM to GDlb. Consequently, overall recovery after all procedures through the gel filtration was about two times higher as compared with that through the dialysis.

Lactones in the neutral lipid fraction.—In the neutral lipid fraction which passed through a DEAE-Sephadex A-25 column, ganglioside lactones that had lost negative charges of carboxyl group by formation of inner esters may be present. The standard GM4LMs were recovered into the fraction eluted with 7:3 $CHCl_3$–MeOH by the first Iatrobeads column chromatography and eluted with 42.5:42.5:15 $CHCl_3$–EtOAc–MeOH by the second chromatography (Fig. 2A). The thin-layer chromatograms of GM4LMs immediately after each step of the analysis is shown in Fig. 2B. The recovery after steps of extraction, ion-exchange chromatography, and concentration was more than 95%. The recovery after each Iatrobeads column chromatography was 90% in either case. The overall recovery of GM4LMs was about 80%.

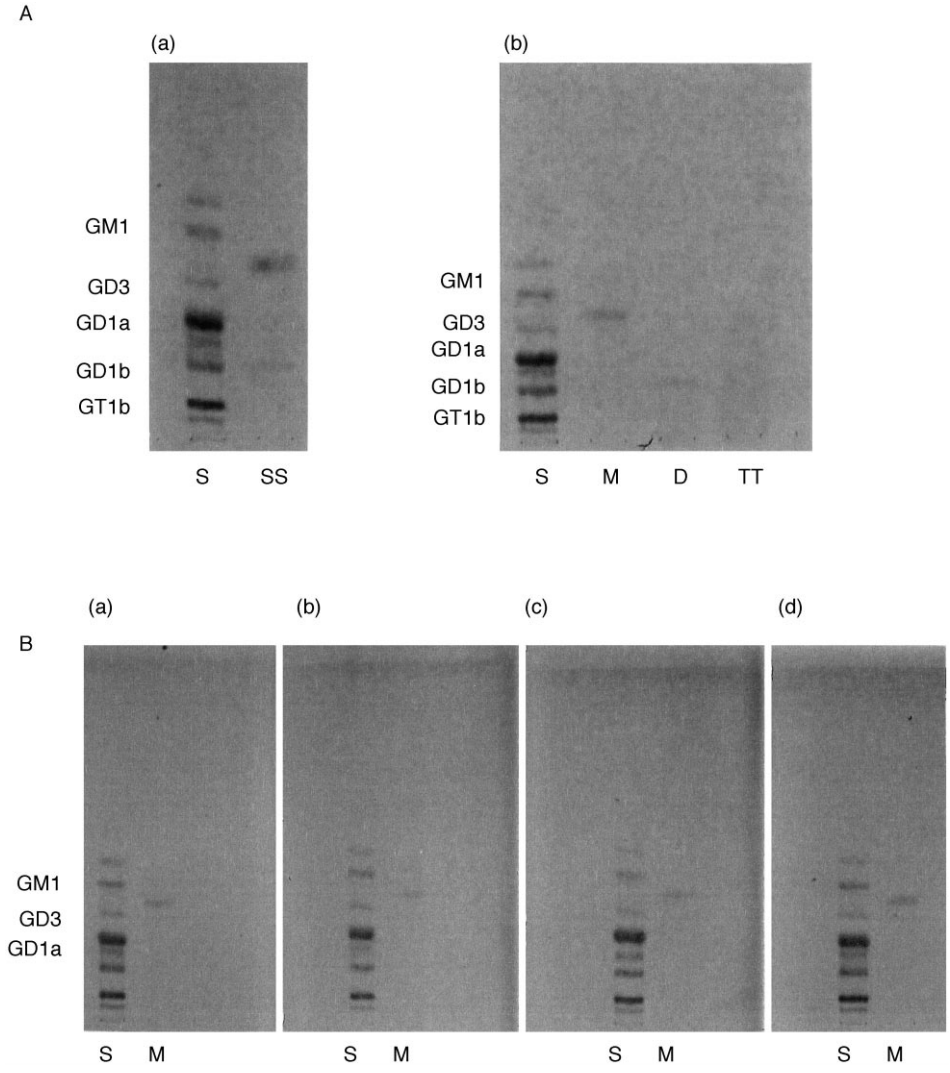

Fig. 1. Analysis of GDlbLM in an acidic lipid fraction. A: stepwise fractionation of GDlbLM and the parent GDlb by DEAE-Sephadex A-25 column chromatography. GDlbLM fraction (GDlbLM 70%, GDlb 30%) was used as a starting sample [SS in (a)]. Fractions, monosialoganglioside (M), disialoganglioside (D), and tri/tetrasialoganglioside (TT) were eluted stepwise using 0.04, 0.08, and 0.2 M NaOAc/MeOH. GDlbLM was clearly separated from GDlb [in (b)]. S, standard ganglioside mixture. B: thin-layer chromatograms of GDlbLM during the preparation procedures. GDlbLM fraction (GDlbLM 70%, GDlb 30%) was used. (a), monosialoganglioside fraction after DEAE-Sephadex A-25 column chromatography; (b), the concentrated sample was applied on a Sephadex LH-20 column after procedure (a); (c), after Sephadex LH-20 column chromatography; and (d), after Iatrobeads column chromatography. S, standard ganglioside mixture. M, monosialoganglioside fraction. In panels A-(a), A-(b), and B, TLC was developed with solvent system I.

Table 1
Ganglioside composition in GDlbLM fraction

	SS	MSFG	DSF	GF
GDlbLM	81	81	77	76
GDlbME	—	19	23	24
GDlb	19	—	—	—
Recovery of	100	73	62	60
GDlbLM	(100)	(75)	(60)	(58)
NeuAc (μg)	330	242	214	210

Per cent of total sialic acid. The data presented is an average value of three experiments, and coefficient of variation was within $\pm 5\%$. SS, starting sample; MSGF, monosialoganglioside fraction; DSF, desalted fraction by Sephadex LH-20 column chromatography; GF, ganglioside fraction after Iatrobeads column chromatography. GDlb in the starting sample was contained in the disialoganglioside fraction. Recovery (in parentheses) was estimated by CD spectrometry

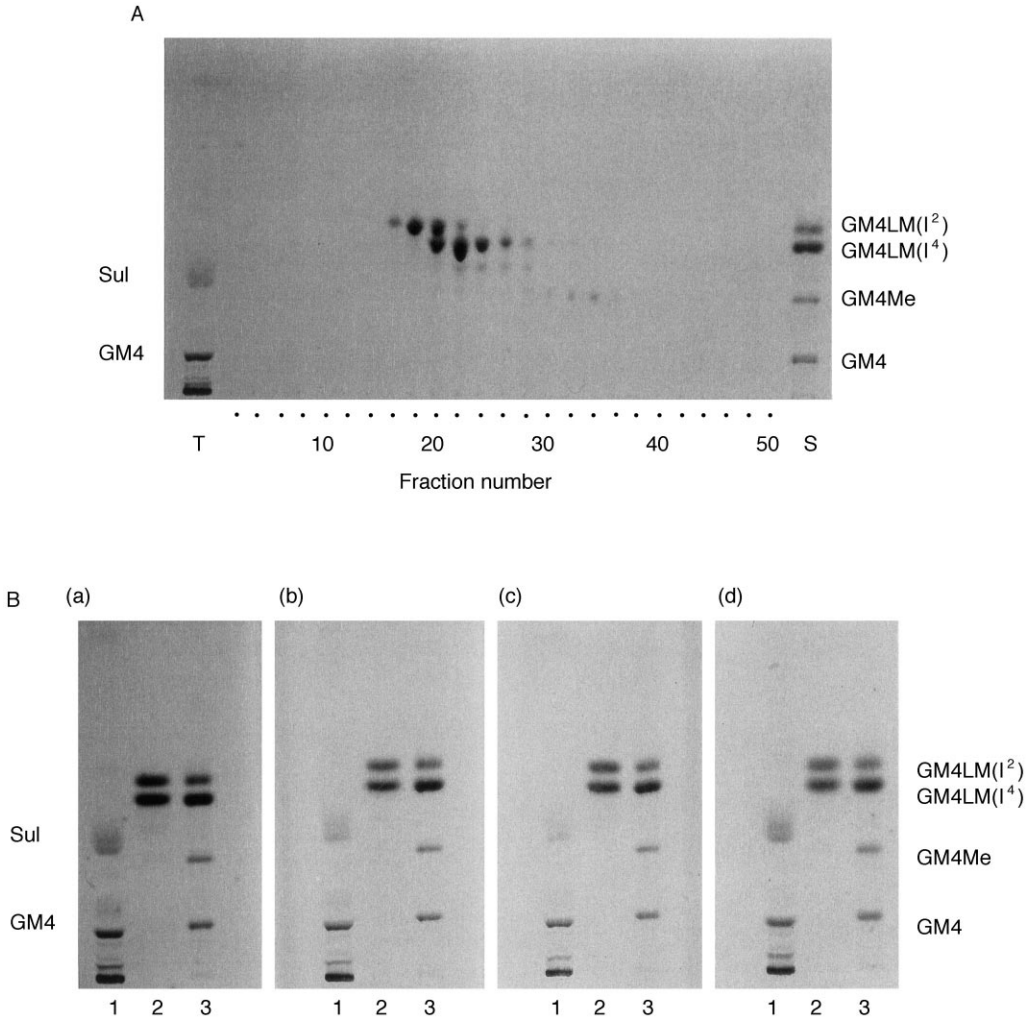

Fig. 2. Analysis of GM4LMs in a neutral lipid fraction. A: fractionation of the GM4LMs fraction by Iatrobeads column chromatography. T, total gangliosides and sulfatide (sul); S, starting sample composed of GM4LMs, GM4Me, and GM4. The sample was fractionated on an Iatrobeads column by a stepwise elution, Fr.1–14, 9:1 $CHCl_3$-MeOH; Fr.15–30, 42.5:42.5:15 $CHCl_3$-EtOAc-MeOH; Fr.31–45, 2:2:1 $CHCl_3$-EtOAc-MeOH; Fr.46–60, 7:3 $CHCl_3$-MeOH, and 2:1 $CHCl_3$-MeOH. GM4 was eluted in Fr.52-60. TLC was developed with solvent system II. B: thin-layer chromatograms of GM4LMs fractions after steps of (a) extraction; (b) DEAE- Sephadex A-25 column chromatography; (c) concentration; and (d) the second Iatrobeads column chromatography. Lane l, total gangliosides and sulfatide (sul); lane 2, GM4LMs fraction; and lane 3, a mixture of standard GM4LMs, GM4Me, and GM4. TLC was developed with solvent system II.

Recovery test of ganglioside lactones.—Prior to the analysis, the overall recovery of ganglioside lactones added to brain tissue homogenate was determined. Ganglioside content in neutral and acidic lipid fractions after DEAE-Sephadex A-25 column chromatography is shown in Table 2. As shown in Fig. 3, gangliosides in the acidic lipid fraction are fractionated clearly into mono-, di-, and tri-/tetrasialoganglioside fractions. Added GD3LM was detected between GM2 and GM3 on the plate. Ganglioside contents after the all procedures were determined by combination of TLC-densitometry and the resorcinol method (Table 2). The overall recovery of GD3LM was 63%. As seen in S–1/10L, it was not easy to detect GD3LM due to the low content. However, application of a large amount of a sample interfered with the optimal resolution of each component on 1D-TLC. Therefore, components in small amount were resolved by 2D-TLC. In this way, alkali-stable gangliosides were located along the diagonal line. The compounds, generated from alkali-labile gangliosides by the intermediate ammonia treatment are detected lying off this line [18]. As shown in Fig. 4, two resorcinol positive spots (a) and (b), generated from the alkali-labile gangliosides, were detected only in the samples to which GD3LM had been added (S–L and S–1/10L). This indicates that

Table 2
Ganglioside contents in neutral lipid fraction and mono-, di-, and tri/tetrasialoganglioside fractions of cerebral gray matter of Minke whale to which GM4LMs, GD3LM and their parent gangliosides were added

	T	S–L			S–1/10L			S–P			C		
SS	2280	2630			2310			2710			2220		
NLF	110	300			130			140			110		
		M	D	TT	M	D	TT	M	D	TT	M	D	TT
GM4	147	194	—	—	165	—	—	339	—	—	144	—	—
GM3	7	7	—	—	5	—	—	10	—	—	7	—	—
GD3LM	—	66	—	—	7	—	—	—	—	—	—	—	—
GM2	32	41	—	—	40	—	—	43	—	—	32	—	—
GM1	125	134	7	—	121	12	—	136	—	—	138	10	—
GD3	68	8	86	—	8	64	—	53	157	—	4	64	
GD1a	442	—	433	12	—	436	10	—	402	—	—	414	11
GD1b	364	—	286	61	—	299	55	—	281	61	—	296	44
O-Ac-GTlb	173	—	—	163	—	11	151	—	5	149	—	9	141
GTlb	434	—	—	412	—	—	413	—	—	389	—	—	411
GQlb	65	—	—	58	—	—	69	—	—	53	—	—	75
NeuAc	1886	1971			1866			2079			1799		

Ganglioside sialic acids were determined by the resorcinol method and TLC-densitometry. The data presented is an average value of three experiments, and coefficient of variation was within ±10%. SS, starting sample; S–L, lactone fractions of GM4LMs (GM4LMs 180 μg, GM4 60 μg) and GD3LM (GD3LM 110 μg, GD3 27 μg) were added to 25 ml of homogenate (0.1 g ml^{-1}) of Minke whale cerebrum; S–1/10L, 1/10 volumes of lactone fractions were added; S–P, parent gangliosies GM4 (290 μg) and GD3 (180 μg) were added. NLF, Neutral lipid fraction; NeuAc, total sialic acid after Iatrobeads column chromatography. M, monosialoganglioside fraction; D, disialoganglioside fraction; TT, tri/tetraganglioside fraction.

the spots are generated from GD3LM. In the first run, the spots comigrated with GM3 and in the second run, faster (b) and slower (a) spots were separated. According to the report by Riboni et al. [19], the spots (b) and (a) were assigned as GD3-amide and GD3, respectively. A relative concentration of GD3LM to GM1 was determined on a 2D-TLC plate by densitometry and the content of GD3LM was calculated based on the content of GM1 in the total ganglioside preparation (Table 3). The two spots were not detected in the sample S-P and in the control sample. This indicates that lactones are not formed from their parent gangliosides during this analytical method. The overall recovery of GD3LM was about 60%.

As shown in Fig. 5a, in the neutral lipid fractions of samples (S–L and S–l/10L) in which ganglioside lactones were added, two resorcinol positive components were detected. The components were alkali-labile derivatives of GM4 but not those of GD3, because each component changed to GM4 after the mild alkali-treatment (Fig. 5b). The faster and slower migrating gangliosides were assigned as GM4LMs and GM4Me, respectively, according to our previous paper [9]. Contents of GM4LMs and GM4Me were 30 μg and 100 μg, respectively. In the preliminary analysis described above, such a large amount of GM4Me was not found. Since lipids such as cholesterol, PC, PE, sphingomyelin, and cerebroside may amount to 70% of total lipids in brain tissue [20], smooth evaporation of organic solvents was hindered by a vigorous bubbling, even after addition of toluene. Prolonged time of evaporation may cause formation of the large amount of GM4Me. As in the analysis of the acidic lipid fraction, however, the two species of alkali-labile gangliosides were not detected in S–P and the control sample. Considering the formation of methyl esters of GM4, GD3, and GDlb, these phenomena show that methyl esters of gangliosides were formed more easily from lactones than from the parent gangliosides. However, lactones were not formed in this analytical procedures. This point is important in the analysis of ganglioside lactones. The natural occurrence of ganglioside lactones *in vivo* has been the subject of a long debate. However, the above results provide basic evidence that lactones found in the ganglioside preparations are naturally occurring components in cells and tissues.

Ganglioside lactones in the brain tissues.—The 2D-TLC of monosialoganglioside fraction of cerebellar gray matter of Minke whale is shown in Fig. 6. Spots a and b generated from GD3LM by the intermediate ammonia treatment are found in line with GM3. Therefore, densitometric determination of GD3LM was performed based on the

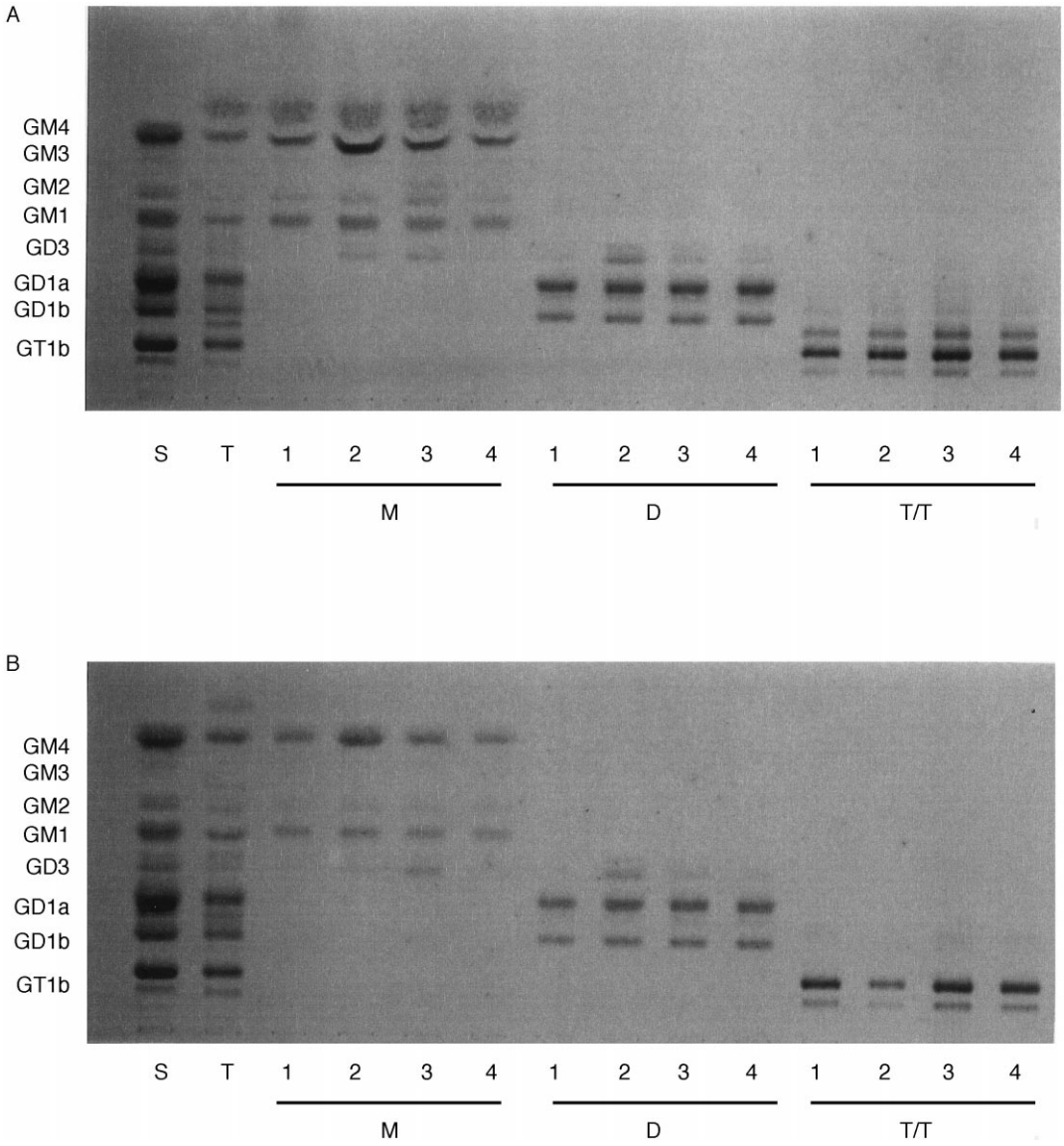

Fig. 3. Thin-layer chromatograms of fractionated gangliosides. S, Ganglioside fraction of cerebral white matter of Minke whale; T, total gangliosides of cerebrum of Minke whale used in this experiment; lane 1, control (S–C); lane 2, sample to which parent ganglioside GM4 (290 μg) and GD3 (180 μg) were added (S–P); lane 3, sample to which lactone fractions of GM4LMs (GM4LMs 180 μg, GM4 60 μg) and GD3LM (GD3LM 110 μg GD3 27 μg) were added (S–L); lane 4, sample to which 1/10 volumes of lactone fraction were added (S–1/10L). M, Monosialoganglioside fraction; D, disialoganglioside fraction; T/T, tri/tetrasialoganglioside fraction. A, before mild alkali- treatment; B, after mild alkali-treatment. TLC was developed with solvent system I.

content of GM3. Concentration of GD3LM in the tissue was $0.005 \pm 0.001\%$ of total ganglioside (three brains), as little as 1/1000 of the parent ganglioside. The most conspicuous feature is that GD3LM is found only in cerebellar gray matter. Concentration of GD3 were around 5% in all tissues analyzed in this study (Table 4). This suggest strongly that GD3LM is specifically abundant in cerebellar gray matter of Minke whale and is an intact component but not an artifact.

Resorcinol positive components found in the neutral lipid fraction are GM4LMs, because chromatographic behaviours (DEAE-Sephadex A-25 and Iatrobeads columns and TLC) agreed well with those found in bride's whale brain [9]. Table 4 shows the concentration of GM4LMs in each tissue of the Minke whale brain, determined by the resorcinol method. It was reported that GM4 was present in high concentration in Minke whale brain [21]. As shown in Table 4, in both the cerebrum and cerebellum, GM4 contents in the white matter are higher than those of the gray matter. However, the highest ratio of GM4LMs/GM4 was found in the cerebral gray matter.

To examine the effect of the storage condition of tissues and the ganglioside preparations on the artificial formation of lactones, samples stored under the following conditions were analyzed: (i) tissues at −80 °C for 1 month; (ii) tissues at −20 °C for 20 days; (iii) tissues at 4 °C for 7 days, and (4) total lipid extract in the mixture of $CHCl_3$, MeOH and H_2O at 20 °C for 7 days. It is noticeable that GD3LM was detected only in cerebellar gray matter after the storage under the above conditions.

Gangliosides are assumed to play a role in a variety of cell surface events such as specific recognition of external ligands and biotransduction of membrane mediated information [22]. Sialic acid and/or its carboxyl group of gangliosides may play a fundamental role in these events. By lactonization, negative charges are reduced without changing the number of sialic acid residues exposed on the cell surface. This type of modification of gangliosides may regulate cellular responses via gangliosides. The presence of ganglioside lactones have been demonstrated in adrenal tissue and melanoma cell (GM3 lactone) [23], brain tissues (GDlb lactone and GM4 lactones) [9,19]. However, the natural occurrence of ganglioside lactones has been the subject of long debate, because lactonization may occur under the conditions used for ganglioside preparation and storage. As described above, we improved an analytical method to overcome the doubt on the natural occurrence of ganglioside lactones. Through the analysis, lactones decomposed to their parent gangliosides and/or methyl esters and were recovered in about 60% yield, but no newly formed lactones could be detected.

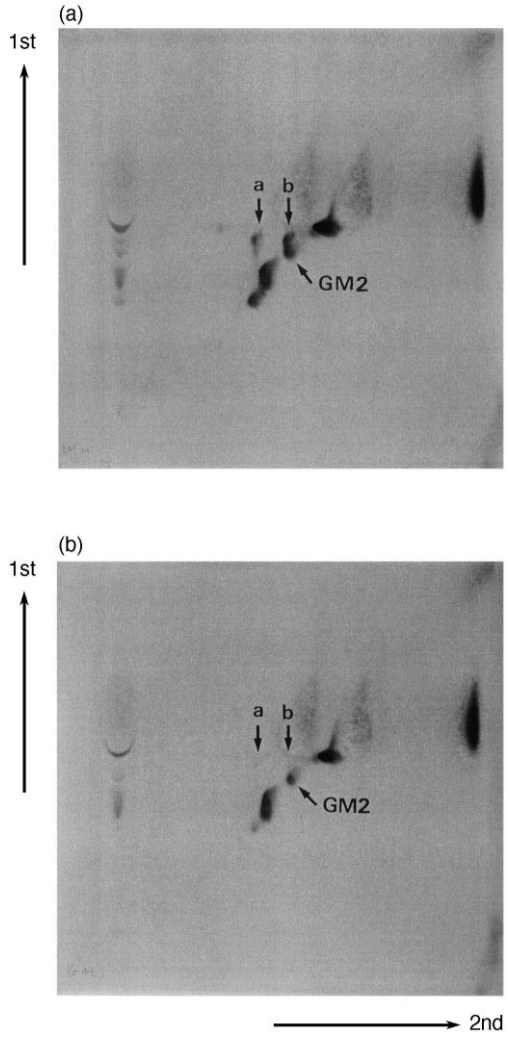

Fig. 4. Two-dimensional thin-layer chromatograms of (a), S–L and (b), S-1/10L. Gangliosides were chromatographed with solvent system I in both directions. Gangliosides developed in the first direction were subjected to an alkali-treatment with ammonia vapor before the second run. Spots a and b are GD3 and GD3-amide, respectively, which are derived from GD3LM added to the homogenate of Minke whale cerebrum by the alkali-treatment.

In this study, GM4LMs and GD3LM in Minke whale brain were determined quantitatively. It was found that the presence of the parent gangliosides is essential for the occurrence of ganglioside lactones but the contents of the ganglioside lactones are not proportional to the content of the parent gangliosides in each tissue. These results suggests strongly that the ganglioside lactones are intact components but not artifacts.

3. Experimental

Ganglioside extraction.—Gangliosides were prepared by the method of Momoi et al. [24] from the

Table 3
Ganglioside composition of Minke whale brain

	Cerebrum		Cerebellum	
	GM	WM	GM	WM
GM4	0.6	29.4	7.2	29.5
GM3	0.8	2.1	1.6	1.1
GM2	2.7	7.3	2.4	4.2
GM1	11.0	8.8	4.2	5.6
GD3	3.5	4.5	5.0	7.9
GD1a	23.9	14.9	10.1	10.1
GT1a + GD2	5.0	5.0	8.1	4.0
GD1b	17.8	11.6	19.6	13.2
GT1b	22.3	12.7	24.0	17.6
GQ1b	5.7	4.3	14.1	6.5
NeuAc(μg/g)	997	769	682	642

GM, gray matter; WM, white matter. Per cent of total sialic acid. The data is an average value of three brains, and coefficient of variation was within ±10%.

whole brain of Minke whale (*Balaenoptera acutorostrata*) which was caught during the sighting and sampling surveys in the 1990/1991 cruise. Each ganglioside was TLC-densitometrically pure. The gangliosides were stored at −80 °C before use.

Ganglioside lactones.—GM4 lactones and mono- and di-lactones of GD3 and GDlb were prepared from their parent gangliosides (GD3 and GDlb) in glacial AcOH [23]. Each ganglioside lactone was purified on an Iatrobeads (6RS-8060, Iatron, Tokyo) column by a stepwise elution using the following solvent systems: 42.5:42.5:15 and 2:2:1 $CHCl_3$–EtOAc–MeOH [9] and followed by 7:3, 65:35, 3:2, and 1:1 $CHCl_3$–MeOH. The ganglioside lactones were stored at −80 °C before use.

Analytical methods.—Total lipids were obtained from cerebral and cerebellar gray matters and white matters (1–3 g wet) by two successive extractions with 10 vol each of 2:1 $CHCl_3$–MeOH and 4:8:3 $CHCl_3$–MeOH–H_2O [25]. The combined extract was applied on a DEAE-Sephadex A-25 column and separated into neutral lipid and acidic lipid fractions. The latter was further fractionated into monosialo-, disialo-, and tri-/tetrasialo-ganglioside fractions by a stepwise elution with 5 vol each of 0.04, 0.08, and 0.2 M NaOAc–MeOH. Each ganglioside fraction was concentrated at 37 °C by a rotary evaporator. The residue was dissolved in 10–20 mL of MeOH and was chromatographed on a Sephadex LH-20 column (1.6×40 cm) with MeOH as an eluent to desalt. Each 4 mL fraction was collected and analyzed by TLC. Desalted ganglioside fraction was concentrated at 37 °C by a rotary evaporator and dissolved in 5 mL of 85:15 $CHCl_3$–MeOH. The solution was applied on an Iatrobeads column (1.6×5 cm). The column was washed with 10 vol of 4:1 $CHCl_3$–MeOH and then gangliosides were eluted with 10 vol of 1:2 $CHCl_3$–MeOH.

In the neutral lipid fraction, the solvent system was a mixture of $CHCl_3$, MeOH, and H_2O. To

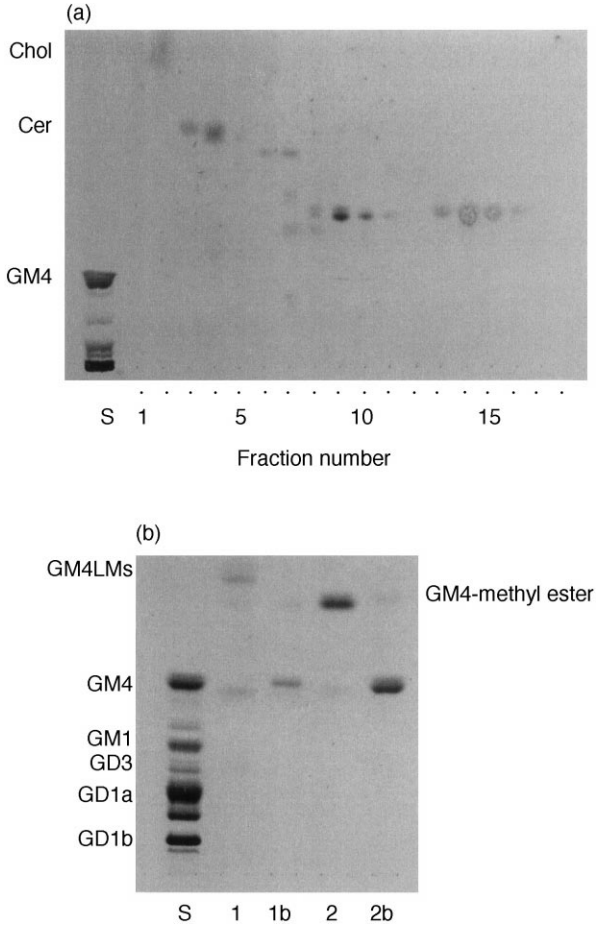

Fig. 5. Analysis of GM4LMs in a neutral lipid fraction of Minke whale brain. a: fractionation of GM4LMs fraction, eluted with 7:3 $CHCl_3$-MeOH from the first Iatrobeads column, by the second latrobeads column. Fractions eluted with 42.5:42.5:15 $CHCl_3$-EtOAc-MeOH (Fr.1–7) and 2:2:1 $CHCl_3$-EtOAc-MeOH (Fr.8–15) were developed with solvent system II. GM4LMs were eluted in Fr.6 and 7 and GM4Me was eluted in Fr.9 and 10. b: thin-layer chromatogram of GM4LMs and GM4Me purified by the second Iatrobeads column. S, Gangliosides of cerebral white matter of Minke whale; lane 1, GM4LMs; lane 1b, GM4LMs after the mild alkali-treatment; lane 2, GM4Me; lane 2b, GM4Me after the mild alkali-treatment. Gangliosides were chromatographed with solvent system I.

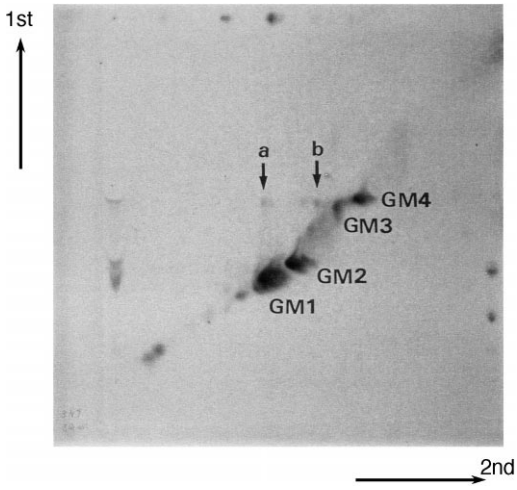

Fig. 6. Two-dimensional thin-layer chromatogram of monosialoganglioside fraction of cerebellar gray matter of Minke whale brain. In the first and second directions, gangliosides were chromatographed with solvent system I. Gangliosides developed in the first dimension were subjected to an alkali-treatment with ammonia vapor before the second run. Spots a and b are identified as GD3 and GD3-amide, respectively, which originated from GD3LM naturally occurring in cerebellar gray matter by the alkali-treatment.

Table 4
Content of GM4 lactones in brain tissues in Minke whale

Tissue	Tissue wt. (g)	Total NeuAc content (μg)	GM4 conc. (%)	GM4 content (μg)	GM4LMs content (μg)	GM4LMs/GM4 (%)
Cerebrum						
Gray matter	8.5	8030	6.0	480	1.8	0.39
White matter	12.5	7950	29	2300	2.4	0.1
Cerebellum						
Gray matter	8.1	5500	7.2	396	n.d.	—
White matter	11.5	7500	29	2180	5.0	0.23

Contents of total NelAc and GM4LMs were determined by the resorcinol method. The content of GM4 was calculated from the total NeuAc content and the concentration of GM4 determined by TLC-densitometry. The data is an average value of three brains, and coefficient of variation was within $\pm 10\%$. About 10 g of each brain tissue was used

prevent hydrolysis of the lactone linkage during the concentration, rotary evaporation was carried out with occasional addition of toluene as an azeotropic reagent for water, until organic solvents were removed almost completely. Residual H_2O was then removed by lyophilization. The neutral lipids were fractionated through two successive Iatrobeads column chromatography. The residue was dissolved in 95:5 $CHCl_3$–MeOH and applied on an Iatrobeads column. Lipid fractions were eluted by successive elution with 5 vol each of 95:5, 9:1, 7:3, 1:1, and 1:2 $CHCl_3$–MeOH. Each fraction was concentrated and dissolved in 95:5 $CHCl_3$–MeOH. The solution was applied on another Iatrobeads column and lipids were fractionated by stepwise elution with 5 vol each of 95:5 and 9:1 $CHCl_3$–MeOH, 42.5:42.5:15 and 2:2:1 $CHCl_3$–MeOH, and 7:3, 1:1, and 1:2 $CHCl_3$–MeOH. Five mL each of the eluate was collected continuously and the lipid composition was monitored by TLC.

Ganglioside compositions of the acidic and the neutral lipid fractions were determined by TLC-densitometry after separation by lD- and/or 2D-TLC. It was confirmed that no artifact lactone was formed through this method, even using a large amount (1 mg as sialic acid) of each authentic ganglioside (GM4, GM1, GD3, GD1a, GD1b, and GT1b).

Recovery test of ganglioside lactones.—Cerebrum (13 g) of Minke whale was homogenized in 130 ml of 2:1 $CHCl_3$–MeOH. In each experiment, 25 mL of the homogenate was used. Total ganglioside composition was analysed by a usual method [17]. Three types of starting samples, S–L, S–1/10L, and S–P, were prepared as follows: into 25 mL each of the homogenate, (1) S–L, GM4LMs fraction (GM4LMs 180 μg and GM4 60 μg) and GD3LM fraction (GD3LM 110 μg and GD3 27 μg; (2) S–1/10L, 1/10 quantity of the lactone fractions of S–L; and (3) S–P, parent gangliosides (GM4 290 μg and GD3 180 μg) was added. Analysis of the three samples along with a control (C) were performed, in parallel, by the improved method from the first extraction step.

Thin-layer chromatography.—In one-dimensional TLC, a small amount (about 1 μg as sialic acids) of the sample solution was applied on a high performance thin layer plate (HPTLC, silica gel 60, E. Merck, Darmstadt). Chromatography was performed in solvent system I, 6:4:1 $CHCl_3$–MeOH-H_2O containing 0.2% $CaCl_2$ or solvent system II, 25:25:25:10:9 n-ProOH–EtOAc–$CHCl_3$–MeOH-H_2O. In two-dimensional TLC, solvent systems I and II were used for the first and second runs, respectively. Alkali treatment of gangliosides were performed directly on the TLC plate with ammonia vapor between the first and the second runs [18]. Gangliosides were visualized by the resorcinol reagent [26]. The ganglioside composition was determined by densitometry using a Shimadzu CS-910 densitometer equipped with a Hewlett-Packard 3390A integrator.

CD spectromery.—CD spectra in the range of 200–300 nm were obtained on a J-20 Spectropolarimeter (JASCO, Tokyo, Japan) using 2 mm and 1 cm cells. Ellipticity difference at 235 nm was obtained to estimate concentrations of the lactones [10].

Mild alkali-treatment.—Mild alkali treatment of ganglioside lactones was carried out by the method of Ueno et al. [27].

Colorimetric method.—Sialic acid in the ganglioside molecules was determined by the resorcinol-HCI method [26] as modified by Miettinen and Takki-Luukkainen [28].

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