Characteristic distribution of glycolipids in gadoid fish nerve tissues and its bearing on phylogeny

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Abstract Glycolipids were isolated from nerve tissues of gadoid fishes including Alaskan pollack and Pacific cod. Their chemical structures were determined by gas–liquid chromatography and gas chromatography–mass spectrometry, and their constituents were analyzed in detail and compared with those of glycolipids from other fish groups. The results revealed that gadoid fish nerve membranes contain peculiar glycolipid molecular species that are distinctly different from those in other teleostean fishes and higher vertebrates. The mole percentage ratio of the four major glycolipids (cerebroside–sulfatide–galactosylglyceride–sulfogalactosylglyceride) was 48:12:25:15, indicating profound accumulation of glycoglycerolipids. Galactosylglyceride and sulfogalactosylglyceride were primarily of the diacyl type (>90%), the major fatty acids being 16:0 and 18:1. An abundance of glucocerebroside (25 to 55% of cerebroside) and its fatty acid ester (37 to 47% of ester cerebroside) was noted. Cerebroside and sulfatide were characterized by the absence of hydroxy and odd numbered fatty acids, and 24:1 acid was a predominant component of both glucocerebroside and galactocerebroside. Subcellular fractionation revealed that myelin membranes comprised such unusual glycolipid constituents as those seen in whole nerve tissues. A vertebrate whose nerve membranes consist of such peculiar glycolipid molecules has not previously been reported. The characteristics of the glycolipid composition in gadoid fishes are discussed in relation to myelin functions, physicochemical properties of nerve membranes, and the phylogenic significance of this fish group. **—Tamai, Y., H. Kojima, S. Saito, K. Takayama-Abe, and H. Horichi.** Characteristic distribution of glycolipids in gadoid fish nerve tissues and its bearing on phylogeny. *J. Lipid Res.* 1992. **33:** 1351–1359.

Supplementary key words cerebroside • ester cerebroside • sulfatide • galactosylglyceride • sulfogalactosylglyceride • myelin • phylogeny • fish brain

Glycolipids represent one of the major components of membrane lipids in the vertebrate nervous system, and their composition is distinctly different in many ways from that in systemic organs or plasma (1, 2). The characteristics of the composition of glycolipids, which are particularly abundant in myelin membranes, generally observed in the brains of higher vertebrates are: *1)* glycosphingolipids are primarily the major class of glycolipids, glycoglycerolipids being very minor components; *2)* glycosyl ceramide and its sulfuric acid ester contain galactose as the sole sugar; and *3)* brain galactosphingolipids contain high proportions of long-chain fatty acids, predominantly C24, and also high proportions of α-hydroxy fatty acids (3).

Previously, we surveyed the phylogenic changes in the ceramide moieties of myelin glycolipids in vertebrates (4). However, the molecular composition and distribution of glycolipids in lower vertebrate nerve tissues have not been sufficiently investigated. The fishes include numerous groups extremely diverse in their habits and the level of development of their nervous systems (5, 6). We can expect, therefore, that systematic studies on the glycolipid constituents of the fish nervous system will yield useful information for understanding the chemical evolution of the nervous system and for elucidating the functional roles of individual glycolipids. In the course of a comparative study on glycolipids of various fish brains, we found that the group of fish called the Gadiformes exhibits a unique distribution of glycolipids that is distinctly different from the general pattern in the vertebrate nerve tissues described above (7). The present paper reports the detailed characterization as well as the systematic distribution of molecular species of glycolipids in the nervous system of Alaskan pollack, a fish in the order 'Gadidae', compared with those of fishes in other orders. The glycolipids inves-

Abbreviations: CMH (cerebroside), galactosyl ceramide (galactocerebroside) or glucosyl ceramide (glucocerebroside); ester CMH, cerebroside fatty acid ester; CSE (sulfatide), cerebroside sulfuric acid ester; MGG (monogalactosyl glyceride), alkylacyl- or diacylgalactosyl glycerol; SGG, sulfate ester of galactosyl glyceride (alkylacyl or diacyl type); GC–MS, gas chromatography–mass spectrometry; IR, infrared; TMS, trimethylsilyl; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

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tigated in this study are those comprising "Folch's lower phase lipids," which represent myelin glycolipids.

MATERIALS AND METHODS

Fishes

Alaskan pollack, *Theragra chalcogramma*, were caught in the northern Pacific Ocean, shipped under ice-cold conditions, and supplied through a local fishery. The brain, olfactory nerve, optic nerve, and spinal cord were separated and kept frozen until analysis. The brains of other fishes were also used for comparison: Pacific cod, *Gadus morrhua macrocephalus*; moridfish, *Lottera maximowiczi*; Japanese goosefish, *Lophius litulon*; and common carp, *Cyprinus carpio*. All fishes used were adult, and either alive or frozen. The mean brain weights were 550 mg for Alaskan pollack, 590 mg for Pacific cod, 435 mg for moridfish, 270 mg for goosefish, and 620 mg for carp.

Authentic glycolipids

The following glycolipids, used as references, were prepared in our laboratory: ceramide-1-O-β-galactoside (galactocerebroside), cerebroside 3-sulfate (sulfatide), and cerebroside fatty acid ester (ester cerebroside) from bovine brain; ceramide-1-O-β-glucoside (glucocerebroside) from Tay-Sachs' brain; and 1,2-diacyl-3-O-β-D-galactopyranosyl-*sn*-glycerol (galactosyldiacyl glycerol) from spinach leaves. 1-O-Alkyl-2-O-acyl-3-O-β-D-(3' sulfo)galactosyl-*sn*-glycerol (seminolipid) from boar testis and 1-O-α-D-galactopyranosyl-*sn*-glycerol prepared by Dr. B. Wickberg (University of Lund, Sweden) were generous gifts from Dr. I. Ishizuka (Teikyo University, Tokyo).

Extraction and purification of glycolipids

The total weights of Alaskan pollack nerve tissues used for lipid extraction were 190 g brain, 8 g olfactory nerve, 35 g optic nerve, and 47 g spinal cord. The pooled brain weights of other fishes were about 2 to 5 g. Nerve tissues were extracted with 20 volumes of chloroform–methanol 2:1 (by vol), 10 volumes of the same solvent, and then 10 volumes of chloroform–methanol–water 1:2:0.3, successively, as described previously (8). The total lipids dissolved in chloroform–methanol 2:1 were partitioned against aqueous 0.88% KCl (9), and the resultant lower phase lipids were chromatographed on a column of Florisil (60–100 mesh; Floridin Co.). Glycolipid fractions were pooled and then applied to a column of DEAE-Sepharose (Pharmacia Fine Chemicals), acetate form, to separate neutral glycolipids from acidic glycolipids. The neutral glycolipid fractions thus obtained were further chromatographed on a column (1.7 × 27 cm) of Iatrobeads (Iatron Co.), which was eluted with 3 column volumes each of chloroform–methanol 95:5 and chloroform–methanol–water 90:12:1 in a stepwise manner, whereby less polar glycolipids and cerebroside (CMH) were efficiently isolated (7). Acidic glycolipid fractions were applied to an Iatrobeads column (8 mm × 45 cm) and eluted with a linear gradient of chloroform–methanol–water 93:7:0.5 to 69:29:2, using two glass reservoirs, respectively, containing 250 ml of the former solvent and 285 ml of the latter solvent, according to a modification of the method of Ishizuka et al. (10). Under these chromatographic conditions, sulfogalactosyl glyceride (SGG) and sulfatide (CSE) were fairly well purified (7). When necessary, the chromatography on an Iatrobeads column was repeated to obtain finally purified neutral or acidic glycolipids. Glucocerebroside and galactocerebroside were isolated from cerebroside as described (11, 12).

Compositional analysis

The amounts of glycolipids were determined by the anthrone sulfuric acid method (13). Sulfated glycolipids were also assayed by two methods using the azur A (14) and sodium rhodizonate (15) reagents.

Infrared (IR) absorption spectrometry of glycolipids was performed with a JASCO DR-81 Infrared Spectrophotometer equipped with a diffuse reflectance attachment (Japan Spectroscopic Co.).

For GLC analyses of glycosphingolipid constituents, the purified materials were hydrolyzed with 3% HCl in methanol as described (8). The hexoses and fatty acids (amide-linked) were analyzed by GLC on a capillary column as their trimethylsilyl (TMS) derivatives and methyl esters, respectively (16). The ester-linked fatty acids of glycolipids were methylated by incubation with 0.1 M sodium methoxide in methanol and then analyzed by GLC. The long-chain base composition of glycosphingolipids, as the corresponding aldehydes (17), was determined by GLC after hydrolysis of the materials with aqueous methanolic HCl as described (18).

For determination of glycoglycerolipid constituents, appropriate amounts of materials were incubated with 0.1 M methanolic NaOH at 40°C for 60 min. The fatty acid methyl esters were analyzed by GLC. The deacylated glycoside materials were separated by TLC with a solvent system of chloroform–methanol–water 65:25:4. The bands corresponding to authentic alkylgalactosyl glycerol and α galactosyl glycerol, which were visualized with iodine vapor, were scraped from the plate and extracted. In some experiments, the deacylated materials were separated by the partition method of Folch, Lees, and Sloane Stanley (9), galactosyl glycerol and alkylgalactosyl glycerol being recovered in the upper and lower phases, respectively. The isolated materials were hydrolyzed with 3% methanolic HCl at 100°C for 3 h. The reaction mixtures were then neutralized with silver carbonate, mannitol being added as an internal standard, and then evaporated to dryness. The dried residues containing methylated sugars and alkylglyceryl ethers were converted

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to the respective TMS derivatives with a mixture of pyridine and trifluoro-N, O-bistrimethylsilyl acetamide (1:2) (10), and then analyzed by GLC on a column of 4% OV-101. The ratio of the alkylacyl form to the diacyl form was determined by comparison of the amount of galactose with that of mannitol in the materials derived from alkylacylgalactosyl glycerol and diacylgalactosyl glycerol, respectively.

Solvolysis of the sulfate esters was carried out essentially as described previously (10). This treatment effectively removed sulfate esters from sulfated glycolipids.

GC–MS analysis

Alkyl glycerols were extracted from the methanolysates of galactosyl glycerides with diethyl ether for separation from methyl galactose, and then converted to their isopropylidene derivatives by the method of Wood (19). GLC was performed on a column of 1% OV-1 (3 mm × 0.5 m) at 180°C isothermally. GC–MS was performed with a GC–MS 9000B (Shimadzu-LKB, Kyoto). Chromatography was carried out on a column of 1.5% OV-1 (3 mm × 2 m) at 230°C, the carrier gas being He at the flow rate of 30 ml/min. Mass spectra were recorded at an electron energy of 70 eV, a trap current of 60 μA, and an ion source temperature of 270°C.

Preparation of the myelin fraction

The myelin fraction of Alaskan pollack brain was prepared by means of sucrose density gradient centrifugation, essentially according to the procedures of Eichberg, Whittaker, and Dawson (20), and Banik and Davison (21). About 8 to 10 g of brain tissues was subjected to subcellular fractionation. The P1 and P2 myelin layers, separated from nuclei and mitochondria, respectively, were combined, diluted with ice-cold water, and then centrifuged at 13,000 *g* for 15 min to sediment the purified myelin fraction.

RESULTS

General characteristics of the glycolipid composition in gadoid fish nerve tissues

Fig. 1 shows typical TLC profiles of total lipids (Folch's lower phase lipids) from the brains of three fishes of the Gadiformes (Alaskan pollack, Pacific cod, and moridfish) and one of the Lophiiformes (goosefish). Alaskan pollack and Pacific cod brains were characterized by the abundance of two glycolipids comigrating with authentic monogalactosyldiacyl glycerol (MGG) and sulfogalactosyl glyceride (seminolipid) (SGG), in addition to cerebroside (CMH) and sulfatide (CSE), whereas in goosefish brain, glycolipids corresponding to MGG and SGG were barely detectable. MGG and SGG in moridfish were less visible than those in the other two gadoid fishes. It was also

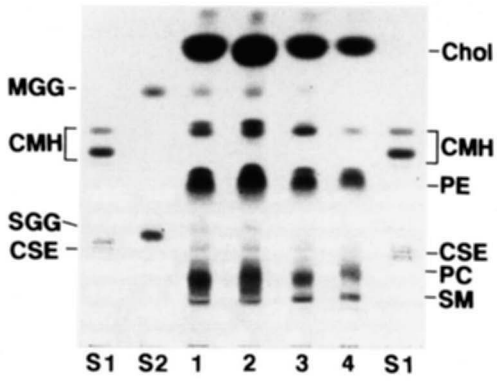

Fig. 1. Thin-layer chromatogram of total lipids from fish brains. The total lipid extracts containing about 10 nmol of phospholipid phosphorus were spotted on a silica gel HPTLC plate (Merck) that was developed with chloroform–methanol–water 65:25:4 (by vol). Lipids were located by spraying the plate with anthrone sulfuric acid reagent, followed by heating. S1, standard cerebroside (CMH) and sulfatide (CSE); S2, standard galactosyldiacyl glycerol (MGG) and sulfogalactosyl glyceride (SGG); 1, Alaskan pollack; 2, Pacific cod; 3, moridfish; 4, Japanese goosefish. Chol, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin. The sources of the standard glycolipids are given under Materials and Methods.

noticed that all four fish brains lacked glycolipids corresponding to CMH and CSE containing hydroxy fatty acids, which are visualized as the lower bands of CMH and CSE, respectively. The two bands observed for cerebroside from Alaskan pollack and Pacific cod are possibly due to the difference in the hexose composition, as shown below.

The five glycolipids comigrating with authentic materials on TLC were isolated as pure fractions from Alaskan pollack nerve tissues (see Figs. 2 and 3) and also from other fish brains. The respective glycolipids isolated from Alaskan pollack were determined as follows.

*Ester cerebroside (ester CMH) (**Fig. 2,** lane 1).* The IR spectrum was almost identical with that of ester CMH from bovine brain (22, 23), showing the presence of carbonyl ester (1738 cm^{-1}), acid amide (1648 cm^{-1}, 1541 cm^{-1}) and sugar (3400 cm^{-1}, 1070 cm^{-1}); the material was shown to be converted to CMH on mild alkaline treatment.

*Galactosyl glyceride (MGG) (diacyl or alkylacyl type) (Fig. 2, lane 2 or 3; **Fig. 3A and B,** lane 1).* The IR spectrum resembled that of MGG (diacyl type) from spinach leaves, showing the presence of carbonyl ester and sugar, but an acid amide bond was absent; galactosyl glycerol and alkylgalactosyl glycerol were produced on mild alkaline treatment of the material, and analyzed by GC–MS, as described below.

Sulfogalactosyl glyceride (SGG) (Fig. 3A and B, lane 3). The IR spectrum was almost identical to that of seminolipid from boar testis (24); the mole ratio of galactose to sulfate was calculated to be 1:1.3 with the anthrone/azur A reagents, and 1:1.0 with the anthrone/sodium rhodizonate reagents. The material was converted to MGG through solvolysis, which was confirmed by the IR spectrum and

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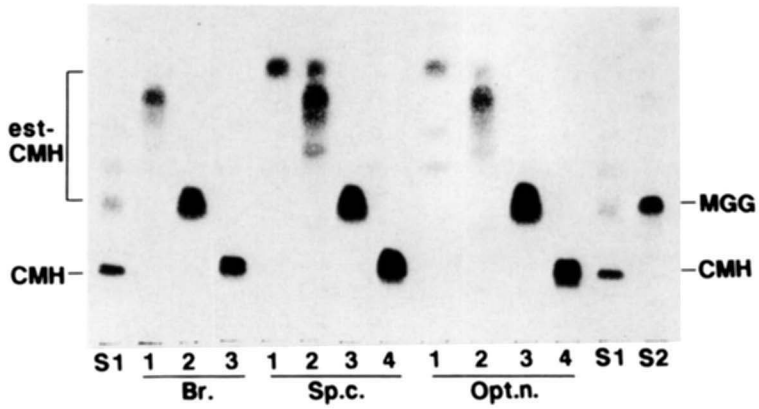

Fig. 2. Thin-layer chromatogram of less polar glycolipids isolated from Alaskan pollack nerve tissues. Solvent system, chloroform-methanol-water 90:12:1. Detection of glycolipids was as indicated in the legend to Fig. 1. Br, brain; Sp.c., spinal cord; Opt.n., optic nerve. S1, standard ester CMH (est-CMH) and CMH; S2, standard MGG; Br. 1, Sp.c. 1 and 2, and Opt.n. 1 and 2: isolated ester CMH; Br. 2, Sp.c. 3, and Opt.n. 3: isolated MGG (diacyl or alkylacyl); Br. 3, Sp.c. 4, and Opt.n. 4: isolated CMH.

TLC. The desulfated material was further degraded to galactosyl glycerol or alkylgalactosyl glycerol by alkaline treatment, which were analyzed by GC-MS, as described below.

CMH and CSE were identified on the basis of their TLC migration and typical IR spectra; the results of compositional analyses are shown below.

Contents and distribution of glycolipids

Table 1 shows that the brains of gadoid fishes such as Alaskan pollack, Pacific cod, and moridfish contain not only glycosphingolipids such as CMH and CSE, but also abundant glycoglycerolipids including MGG and SGG; the mole percentage of the latter accounted for as much as 20-40% of the total glycolipids, in contrast to goosefish and carp, in which glycosphingolipids were major components, with CMH accounting for 60-70% and glycoglycerolipids for less than 10%. In Alaskan pollack nerve tissues, the relative concentrations of glycoglycerolipids were particularly high in brain and olfactory nerve, and ester CMH was most abundant in spinal cord.

Composition of glycosphingolipids

CMH and ester CMH in Alaskan pollack and Pacific cod were found to contain large amounts of glucose as the hexose component, the levels being as high as 55% in olfactory nerve and 25%, at minimum, in optic nerve. In contrast, in goosefish and carp, the glucose content was less than 5% (Table 1). It was noted that ester CMH glucose comprised as much as nearly 40% in brain and 50% in spinal cord.

The fatty acid compositions of CMH and CSE from Alaskan pollack (**Table 2**) and other gadoid fishes (**Table 3**) were characterized by the absence of hydroxy fatty acids. These findings corresponded to TLC results shown in Figs. 1 to 3. The major component was nonhydroxy 24:1, which amounted to nearly 80% of the total CMH fatty acids. On the other hand, carp brain contained significant

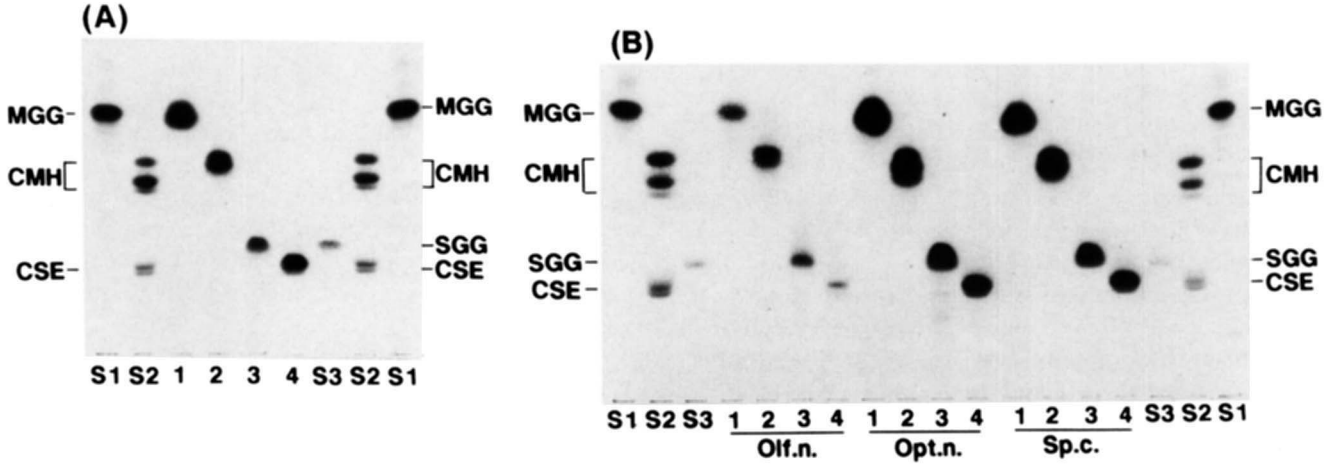

Fig. 3. Thin-layer chromatograms of glycolipids isolated from Alaskan pollack nerve tissues. Plate A, brain; plate B, olfactory nerve (Olf.n.), optic nerve (Opt.n.), and spinal cord (Sp.c.). Solvent system, chloroform-methanol-water 65:25:4. Detection of glycolipids was as indicated in the legend to Fig. 1. S1, standard MGG; S2, standard CMH and CSE; S3, standard SGG. 1, 2, 3, and 4, isolated MGG, CMH, SGG, and CSE, respectively.

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TABLE 1. Contents and distribution of glycolipids in Alaskan pollack and various fish nerve tissues

Lipids	Alaskan Pollack				Pacific Cod	Moridfish	Japanese Goosefish	Common Carp
	Brain	Olfactory Nerve	Optic Nerve	Spinal Cord	Brain	Brain	Brain	Brain
Total lipids (mg/100 mg wet tissue)	11.5	6.3	19.0	17.7	n.d.	8.4	6.1	11.5
Glycolipids (nmol/100 mg wet tissue)								
Ester CMH	9.0 (1)	n.d.	20.3 (1)	39.8 (2)	n.d.[a]	—[b]	—	—
Glc (%)[c]	37.5	n.d.	44.4	47.2	n.d.	n.d.	n.d.	n.d.
MGG	285.4 (25)	272.5 (36)	234.6 (14)	394.5 (20)	115.0 (16)	157.0 (17)	37.0 (8)	12.2 (7)
CMH	556.3 (48)	423.6 (56)	991.0 (59)	1031.2 (52)	422.0 (59)	597.0 (64)	260.0 (58)	135.6 (74)
Glc (%)	33.5	54.9	25.1	44.5	40.0	13.7	4.7	<1
SGG	168.7 (15)	47.7 (6)	226.4 (14)	258.0 (13)	84.0 (12)	32.0 (3)	—	0
CSE	134.6 (12)	18.0 (2)	199.3 (12)	265.7 (13)	98.0 (14)	154.0 (16)	154.0 (34)	36.6 (20)
GGL (%)[d]	39.4	42.0	27.6	32.8	27.7	20.1	8.2	6.6

Data are averages of three or more determinations. Values in parentheses are mole percentages of the individual glycolipids as to the total glycolipids.
[a]Not determined.
[b]Not analyzed due to small amount.
[c]Weight percentage of glucose as to the total hexose (glucose + galactose).
[d]GGL (%), mole percentage of glycoglycerolipids (MGG + SGG) as to the total glycolipids.

amounts of hydroxy and nonhydroxy 24:0 and 24:1 (Table 3). The fatty acid profiles were generally the same as those found in other fish species (7); the content of hydroxy fatty acids amounted to about 10% of the total fatty acids. The isolated glucocerebroside and galactocerebroside showed almost identical fatty acid compositions to that of the total CMH.

Ester CMH was also characterized by the absence of hydroxy fatty acids (**Table 4**). The major components in ester-linked fatty acids were 16:0 and 18:1, and 24:1 was the most abundant in amide-linked fatty acids. Odd-numbered fatty acids were not found in all glycosphingolipids.

The long-chain bases of CMH, ester CMH, and CSE consisted of almost only sphingenine (90 to 95%), sphinganine being a minor component (less than 5%) in all nerve tissues of Alaskan pollack.

Composition of glycoglycerolipids

Both MGG and SGG consisted mainly of the diacyl type in all fishes examined, the alkylacyl type being a very minor component (**Table 5**). The hexose component was solely galactose. The fatty acid compositions of MGG and SGG are shown in **Table 6.** 16:0 and 18:1 were the two major components of MGG from Alaskan pollack and Pacific cod nerve tissues. In carp brain, the amount of 18:1 was lower than in the former two fishes, and amounts of 16:1 and fatty acids with carbon chains longer than 20 were higher. SGG showed a slightly different fatty acid composition from that of MGG, 18:1 amounting to almost 50% of the total and 16:0 acid comprising 20–30%. Both MGG and SGG were highly unsaturated as to their fatty acid compositions.

TMS derivatives of alkylglyceryl ethers were insufficiently separated by GLC on a column of 4% OV-101; in particular, 1-O-alkyl and 2-O-alkyl isomers were not resolved. Therefore, alkylglyceryl ethers were analyzed by GC–MS as their isopropylidene derivatives. MGG and SGG showed similar alkyl glycerol compositions: 1-O-hexadecenyl (16:1), 1-O-hexadecyl (16:0), 1- O-octadecenyl (18:1), and 1-O-tetradecyl (14:0) glycerol being the major components in Alaskan pollack (**Table 7**); 16:0 accounted for 50% in Pacific cod MGG. The profiles of aliphatic chains greatly differed between the alkyl and acyl moieties.

Distribution of glycolipids in myelin of Alaskan pollack brain

As shown by electronmicroscopy, the myelin lamellae were rather thin and loose, compared to those of higher vertebrate brains. The average yield of myelin protein for three preparations was 0.58 mg/g brain, corresponding to 1.0% of the total brain protein. The mole percentage ratio of the four major glycolipids in myelin was characterized by the greater predominance of MGG and SGG than in

TABLE 2. Fatty acid composition of glycosphingolipids from Alaskan pollack nerve tissues

Fatty Acid	Brain		Olfactory Nerve		Optic Nerve		Spinal Cord	
	CMH	CSE	CMH	CSE	CMH	CSE	CMH	CSE
	% of total fatty acid							
16:0	2	3	7	15	7	4	2	4
18:0	7	6	6	11	18	9	10	8
22:0	1	1	1	trace	2	1	1	2
22:1	6	7	5	5	10	12	8	9
24:1	82	82	73	65	61	73	77	76
Others	2	1	8	4	2	1	2	1

Values are averages of two or more determinations by GLC. All fatty acids are nonhydroxy.

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TABLE 3. Fatty acid composition of glycosphingolipids from various fish brains

Fatty Acid	Pacific Cod		Moridfish		Japanese Goosefish		Common Carp			
	CMH	CSE	CMH	CSE	CMH	CSE	CMH(NOH)	CMH(OH)	CSE(NOH)	CSE(OH)
16:0	4	5	2	12	13	16	12	–[a]	22	–
18:0	15	23	1	3	9	13	4	–	7	2
18:1	2	1	–	1	–	–	5	–	5	4
22:0	1	2	4	3	29	26	3	11	3	8
22:1	8	7	9	9	4	3	1	1	2	–
24:0	–	–	–	–	–	–	9	44	9	36
24:1	67	62	87	72	35	33	44	33	23	41
Others	3	–	1	3	10	9	22	11	29	15
NOH[b]	100	100	100	100	100	100	89		90	

Values are averages of two or more determinations by GLC and percentages as to the total nonhydroxy (NOH) or hydroxy (OH) fatty acids. In carp brain, 16:1, 26:0, 26:1, 26h:0 and 26h:1 were also present.

[a]Not detected or negligible amount.

[b]The percentage of total nonhydroxy fatty acids as to the total fatty acids.

brain (**Table 8**), these two glycolipids comprised 60% of the total in myelin (compare with Table 1). In contrast, in carp myelin, glycosphingolipids (CMH + CSE) amounted to more than 90% of the total myelin glycolipids (see values in parentheses in Table 8). Glucocerebroside was abundant also in myelin, as observed in brain. The fatty acid composition of myelin glycolipids almost coincided with that in brain for all four glycolipids.

DISCUSSION

The present study shows that glycolipids in gadoid fish nerve membranes comprise unique molecular species, which are distinctly different from the general composition in the vertebrate nervous system. The major characteristics are *1*) the abundance of glycoglycerolipids such as MGG and SGG; *2*) the absence of hydroxy and odd-numbered fatty acids in glycosphingolipids; and *3*) the abundance of glucocerebroside and the presence of glucocerebroside fatty acid ester. First, we will compare these peculiar findings with data from other vertebrates, and then discuss their phylogenic and physiological significance.

Although glycosylglycerolipids have generally been found in the vertebrate brain (25), their content accounts for only 10% or less of the total glycolipids in rat (26), frog (8), and coelacanth (27) brain. SGG, the alkylacyl type of sulfogalactosylglyceride, was the first characterized after isolation from boar testis (24). The presence of the diacyl type of SGG in brain has been reported so far in a restricted number of vertebrate species, the amount being very low (10, 28–30). A vertebrate species that contains as much MGG and SGG as gadoid fishes has not been reported to date.

The composition of aliphatic moieties of glycosylglycerides varies with the animal species and also with the developmental stage. The diacyl type was almost the only type found in gadoid fishes, while the alkyldiacyl type was found in an amount comparable to that of the diacyl type

TABLE 4. Fatty acid composition of ester CMH from Alaskan pollack nerve tissues

Fatty Acid	Ester-Linked			Amide-Linked		
	Brain	Optic Nerve	Spinal Cord	Brain	Optic Nerve	Spinal Cord
	% of total fatty acids					
16:0	39	26	33	14	5	7
16:1	6	9	8	4	1	1
18:0	11	7	9	15	12	14
18:1	33	42	36	5	1	3
20:1	4	5	6	2	trace	1
22:1	1	1	1	5	7	15
24:1	6	7	4	46	70	55
Others	trace	3	3	9	4	4

Values are averages of two or more determinations by GLC.

TABLE 5. Percentage composition of the alkylacyl and diacyl types of MGG and SGG from fish nerve tissues

Fish	Nerve Tissue	Lipids	Diacyl	Alkylacyl
			% of total	
Alaskan pollack	Brain	MGG	97	3
		SGG	98	2
	Optic nerve	MGG	97	3
		SGG	100	trace
	Spinal cord	MGG	98	2
		SGG	87	13
Pacific cod	Brain	MGG	96	4
		SGG	99	1
Common carp	Brain	MGG	86	14

Lipids were deacylated with methanolic NaOH and then separated by TLC. Deacylated glycosides were hydrolyzed with methanolic HCl and then the contents of liberated methyl galactose were determined by GLC.

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TABLE 6. Fatty acid composition of MGG from fish nerve tissues

Fatty Acid	MGG						SGG			
	Alaskan Pollack				Pacific Cod	Common Carp	Alaskan Pollack			
	Brain	Olfactory Nerve	Optic Nerve	Spinal Cord	Brain	Brain	Brain	Olfactory Nerve	Optic Nerve	Spinal Cord
	% of total fatty acids									
14:0	1	trace	1	1	1	1	1	trace	trace	2
16:0	37	36	24	32	41	43	29	22	19	28
16:1	13	8	14	12	11	20	12	9	15	18
18:0	2	3	4	3	3	7	3	6	3	2
18:1	35	41	42	41	36	11	45	49	48	38
20:1	8	9	8	9	9	5	8	8	8	8
Others	4	3	7	2	trace	13[a]	2	6	7	4

Values are averages of two or three determinations by GLC.
[a]Others in common carp, 14:1, 20:0, 22:0, and 22:1.

in rat (10, 29) and frog brain (8). The distribution of fatty acids and alkylglyceryl ethers of MGG and SGG observed in gadoid fishes was also considerably different from that in rat (10, 28), 18:1 acid and 16:1 ether being rather abundant in gadoid fishes.

Galactosphingolipids, such as CMH and CSE, in the vertebrate nervous system have a high proportion of α-hydroxy fatty acids, and the proportion in total fatty acids has been found to decrease with an animal's position, from top to bottom, on the evolutionary tree (31). A nervous system in which CMH and CSE are lacking in hydroxy fatty acids has so far been reported only for the Urodela (Amphibia) (32, 33). The gadoid fishes are, therefore, the second vertebrate group lacking hydroxy fatty acids in nerve membranes.

Glucocerebroside is barely detectable in the adult vertebrate brain (31). It should be noted that nearly half of CMH was glucocerebroside in Alaskan pollack and Pacific cod nerve tissues. The fatty acid composition of glucocerebroside from gadoid fishes was identical to that of galactocerebroside, C24:1 being predominant. These findings suggest that glucocerebroside is also one of the major structural components of nerve membranes in gadoid fishes.

Ester CMH has been widely found in vertebrates, although only in small amounts, e.g., 0.42 mg/g of brain or 1% of crude sphingolipids, and the only hexose component of ester CMH so far reported is galactose (22, 34). This may be the first report of the accumulation of an acyl ester of glucocerebroside in nerve tissues. Ester CMH, in general, consists of several isomers with different substituted positions of the acyl group on the hexose moiety (22, 35, 36). The results of detailed structural determination will be reported elsewhere.

CMH, CSE, and MGG were found to be exclusively localized in the myelin fraction in mammals (26). The present study shows that myelin membranes of Alaskan pollack brain have unusual glycolipid profiles. Previously, Ki et al. (37) reported that the nerve conduction velocities in salamander and newt, which lack hydroxy fatty acids, were significantly reduced, compared with those in

TABLE 7. Composition of alkylglyceryl ethers of MGG and SGG from Alaskan pollack and Pacific cod brain

Glyceryl Ethers	Alaskan Pollack		Pacific Cod
	MGG	SGG	MGG
	% of total fatty acids		
14:0	16	15	11
14:1	1	1	1
16:0	22	22	53
16:1	39	40	20
18:0	2	2	trace
18:1	21	21	15

Individual components were identified by GC–MS. Values are averages of two or three determinations by GLC.

TABLE 8. Glycolipid composition of myelin of Alaskan pollack brain

	CMH	CSE	MGG	SGG
mole %	26	13	40	21
	(71)	(21)	(7)	(0)
Glucose (%)	44	0	0	0
Fatty acid (%)				
16:0	4	11	41	35
16:1	1	3	11	18
18:0	7	7	4	3
18:1	2	5	33	35
20:1			7	7
22:0	1	1		
22:1	6	8		
24:1	80	64		
Others	trace	1	4	2

Myelin glycolipids were isolated and analyzed by the same procedures as those used for nerve tissues. Values are averages of two determinations. The numbers in parentheses are the values for common carp.

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chameleon, which has hydroxy fatty acids, implying a role of hydroxy fatty acid-containing glycolipids in nerve conduction velocity. The myelin membranes of gadoid fishes, therefore, may be expected to function very differently from those of other vertebrates.

The peculiar glycolipid composition seen in gadoid fish nerve tissues can be interpreted from two viewpoints: phylogeny or taxonomy, and physicochemical properties of glycolipids. Earlier, Greenwood et al. (38) proposed a new classification system for teleostean fishes. According to their conception of the evolutionary relationship of fishes, both the Gadifomes (Alaskan pollack, Pacific cod, and moridfish in this study) and the Lophiiformes (goosefish) are classified as "Paracanthopterygii," and they all lack hydroxy fatty acids, as shown in this study. On the other hand, carp and other fishes, whose brain glycolipids contain hydroxy fatty acids, as far as examined (present study and 7), are all classified separately from the "Paracanthopterygii." Furthermore, only gadoid fish nerve tissues contain particularly high amounts of glucocerebroside. The neurochemical findings in this study appear to be in good agreement with the classification of Greenwood et al. (38), which is based mainly on the morphological characteristics and evolutionary trends of fishes. Previously, Okamura and coworkers (31) suggested that the predominant biosynthesis of galactocerebroside over glucocerebroside in the nervous system was correlated with an evolutionary trend from Prostomia to Deuterostomia. Our findings for gadoid fishes, however, may deviate from their conception.

The physicochemical properties of glycolipid molecules examined by means of differential scanning calorimetry have been shown to differ considerably with glycolipid species (39–41). Either hydroxy or nonhydroy fatty acids in the hydrocarbon portion of glycolipids and either galactose or glucose in the polar head group greatly affect thermotropic behavior, such as the transition temperature and enthalpy of glycolipid-containing liposomes. Glycoglycerolipid-containing liposomes show a lower transition temperature than those containing glycosphingolipids (42, and H. Kojima and Y. Tamai, unpublished data). The melting points of hydroxy fatty acids are considerably higher, e.g., by 10° to 20°C, than those of nonhydroxy fatty acids of the same chain lengths (43). Furthermore, fatty acids of glycolipids from gadoid fish nerve membranes are very much enriched with unsaturated components, whose melting points are considerably lower than those of saturated fatty acids. Taking the present findings together with the reported ones into account, the nerve membranes of gadoid fishes are reasonably considered to be extremely fluid, compared with those of other vertebrates, including other classes of fishes. However, we cannot say at present whether or not such environmental conditions as sea depth and low temperature are related to the characteristic distribution of glycolipids in gadoid fish nerve membranes shown here.

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REFERENCES

1. Ramsey, R. B., and H. J. Nicholas. 1972. Brain lipids. *Adv. Lipid Res.* **10:** 143–232.
2. Sweeley, C. C., and B. Siddiqui. 1977. Chemistry of mammalian glycolipids. *In* The Glycoconjugates. Vol. 1. M. I. Horowitz and W. Pigman, editors. Academic Press, New York. 507–515.
3. Suzuki, K. 1981. Chemistry and metabolism of brain lipids. *In* Basic Neurochemistry, 3rd ed. G. J. Siegel, R. W. Albers, B. W. Agranoff, and R. Katzman, editors. Little, Brown and Company, Boston. 355–370.
4. Tamai, Y. 1985. Evolution of glycolipids in the vertebrate nervous system: phylogenic distribution of myelin glycolipids and its physiological significance (in Japanese). *Seikagaku.* **57:** 1249–1270.
5. McFarland, W. N., F. H. Pogh, T. J. Cade, and J. B. Heiser. 1979. Vertebrate Life. Macmillan Publishing Co., New York. 177–220.
6. Sarnat, H. B., and M. G. Netsky. 1981. Evolution of the Nervous System. Oxford University Press, New York. 3–23.
7. Tamai, Y., H. Kojima, K. Abe, and S. Saito. 1982. Glycolipid composition of fish brains and its bearing on the phylogeny. *In* New Vistas in Glycolipid Research. A. Makita, S. Handa, T. Taketomi, and Y. Nagai, editors. Plenum Press, New York. 315–323.
8. Saito, S., and Y. Tamai. 1983. Characteristic constituents of glycolipids from frog brain and sciatic nerve. *J. Neurochem.* **41:** 737–744.
9. Folch, J., M. Lees, and G. H. Sloan Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
10. Ishizuka, I., M. Inomata, K. Ueno, and T. Yamakawa. 1978. Sulfated glyceroglycolipids in rat brain. Structure, sulfation in vivo, and accumulation, in whole brain during development. *J. Biol. Chem.* **253:** 898–907.
11. Young, O. M., and J. K. Kanfer. 1965. An improved separation of sphingolipids by thin-layer chromatography. *J. Chromatogr.* **19:** 611–613.
12. Kean, E. L. 1966. Separation of gluco- and galactocerebrosides by means of borate thin-layer chromatography. *J. Lipid Res.* **7:** 449–452.
13. Yamakawa, T., R. Irie, and M. Iwanaga. 1960. The chemistry of lipid of posthemolytic residue or stroma of erythrocytes. IX. Silicic acid chromatography of mammalian stroma glycolipids. *J. Biochem.* **48:** 490–507.
14. Kean, E. L. 1968. Rapid, sensitive spectrophotometric method for quantitative determination of sulfatides. *J. Lipid Res.* **9:** 319–327.
15. Terho, T. T., and K. Hartiala. 1971. Method for determination of the sulfate content of glycosaminoglycans. *Anal. Biochem.* **41:** 471–476.

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16. Abe, K., and Y. Tamai. 1982. Simultaneous determination of methyl esters of α-hydroxy and nonhydroxy fatty acids from brain cerebroside by fused-silica capillary gas chromatography. *J. Chromatogr.* **232:** 400–405.
17. Sweeley, C. C., and E. A. Moscatelli. 1959. Qualitative microanalysis and estimation of sphingolipid bases. *J. Lipid Res.* **1:** 40–47.
18. Kojima, H., and Y. Tamai. 1975. Constituents of ceramide monohexoside isolated from equine kidney. *Jpn. J. Exp. Med.* **45:** 231–234.
19. Wood, R. 1967. GLC and TLC analysis of isopropyledene derivatives of isomeric polyhydroxy acids derived from positional and geometrical isomers of unsaturated fatty acids. *Lipids.* **2:** 199–203.
20. Eichberg, J., V. P. Whittaker, and R. M. C. Dawson. 1964. Distribution of lipids in subcellular particles of guinea pig brain. *Biochem. J.* **92:** 91–100.
21. Banik, N. L., and A. N. Davison. 1969. Enzyme activity and composition of myelin and subcellular fractions in the developing rat brain. *Biochem. J.* **115:** 1051–1062.
22. Tamai, Y. 1968. Further study on the faster running glycolipid in brain. *Jpn. J. Exp. Med.* **38:** 65–73.
23. Tamai, Y., T. Taketomi, and T. Yamakawa. 1967. New glycolipids in bovine brain. *Jpn. J. Exp. Med.* **37:** 79–81.
24. Ishizuka, I., M. Suzuki, and T. Yamakawa. 1973. Isolation and characterization of a novel sulfoglycolipid, 'seminolipid,' from boar testis and spermatozoa. *J. Biochem.* **73:** 77–87.
25. Murray, R. K., and R. Narasimhan. 1990. Glycoglycerolipids of animal tissues. *In* Glycolipids, Phospholipids, and Sulfoglycolipids. M. Kates, editor. Plenum Press, New York. 321–361.
26. Nonaka, G., and Y. Kishimoto. 1979. Levels of cerebroside, sulfatide, and galactosyl diglycerides in different regions of rat brain. Change during maturation and distribution in subcellular fractions of gray and white matter of sheep brain. *Biochim. Biophys. Acta.* **572:** 432–441.
27. Tamai, Y., H. Kojima, and K. Abe. 1986. Chemical characterization of the brain of a coelacanth, *Latimeria chalumnae*. *Comp. Biochem. Physiol.* **83B:** 295–299.
28. Levine, M., M. J. Kornblatt, and R. K. Murray. 1975. Isolation and partial characterization of a sulfogalactoglycerolipid from rat brain. *Can. J. Biochem.* **53:** 679–689.
29. Pieringer, J., G. S. Rao, P. Mandel, and R. A. Pieringer. 1977. The association of the sulfogalactosylglycerolipid of rat brain with myelination. *Biochem. J.* **166:** 421–428.
30. Ishizuka, I., and M. Inomata. 1979. Sulfated glycoglycerolipids in rat brain: decrease and disappearance after developmental age. *J. Neurochem.* **33:** 387–388.
31. Okamura N., M. Stokskopf, F. Hendricks, and Y. Kishimoto. 1985. Phylogenetic dichtomy of nerve glycosphingolipids. *Proc. Natl. Acad. Sci. USA.* **82:** 6779–6782.
32. Ramsey, R. B. 1976. Galactolipid content and fatty acid composition of amphibian central nervous system. *J. Neurochem.* **27:** 1559–1661.
33. Ki, P. F., and Y. Kishimoto. 1984. The lipid composition of urodele myelin which lacks hydroxycerebroside and hydroxysulfatide. *J. Neurochem.* **42:** 994–1000.
34. Kishimoto, Y., M. Wajda, and N. S. Radin. 1968. 6-Acyl galactosyl ceramides of pig brain: structure and fatty acid composition. *J. Lipid Res.* **9:** 27–33.
35. Yasugi, E., E. Saito, T. Kasama, H. Kojima, and T. Yamakawa. 1982. Occurrence of 2-O-acyl galactosyl ceramide in whale brain. *J. Biochem.* **91:** 1121–1127.
36. Yasugi, E., T. Kasama, H. Kojima, and T. Yamakawa. 1983. Occurrence of 2-O-acyl galactosyl ceramide in human and bovine brain. *J. Biochem.* **93:** 1595–1599.
37. Ki, P., Y. Kishimoto, E. E., Lattman, E. F., Stanley, and J. W. Griffin. 1985. Structure and function of urodele myelin lacking alpha-hydroxy fatty acid-containing galactosphingolipids: slow nerve conduction and unusual myelin thickness. *Brain Res.* **345:** 19–24.
38. Greenwood, P. H., D. E. Rosen, S. H. Weitzman, and G. S. Myers. 1966. Phyletic studies of teleostean fishes, with a provisional classification of living forms. *Am. Mus. Nat. Hist.* **131:** 339–456 (Article 4).
39. Maggio, B., T. Ariga, J. M. Sturtevant, and R. K. Yu. 1985. Thermotropic behavior of glycosphingolipids in aqueous dispersions. *Biochemistry.* **24:** 1084–1092.
40. Maggio, B., T. Ariga, J. M. Sturtevant, and R. K. Yu. 1985. Thermotropic behavior of binary mixtures of dipalmitoylphosphatidylcholine and glycosphingolipids in aqueous dispersions. *Biochim. Biophys. Acta.* **818:** 1–21.
41. Kojima, H., K. Harada-Yoshikawa, A. Katagiri, and Y. Tamai. 1988. Thermotropic behavior and electronmicroscopic structures of mixtures of gangliosides and dipalmitoylphosphatidylcholine. *J. Biochem.* **103:** 126–131.
42. Endo, T., K. Inoue, and S. Nojima. 1982. Physical properties and barrier function of synthetic glyceroglycolipids. *J. Biochem.* **92:** 953–960.
43. Markley, K. S. 1972. Fatty acids: physical and chemical properties. *In* Biological Data Book. Vol. 2, 2nd edition. P. L. Altman and D. S. Dittmer, editors. Federation of American Societies for Experimental Biology, Bethesda. 300–347.

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