papers and notes on methodology

Determination of peracetylated sulfoglycolipids using the azure A method

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Abstract Several sulfoglycolipids, including sulfogalactosylceramide, sulfolactosylceramide, monosulfogangliotriaosylceramide, monosulfogangliotetraosylceramide, bis-sulfogangliotriaosylceramide, bis-sulfogangliotetraosylceramide, seminolipid, and lysoseminolipid, were assayed by the azure A procedure (Kean, E. L. 1968. *J. Lipid Res.* **9:** 319–327). The color yields were different significantly among each sulfoglycolipid. The accurate determination of monosulfoglycolipids having longer carbohydrate chains than sulfolactosylceramide as well as bis-sulfoglycolipids could not be achieved because of the low color yields, turbidity, and/or formation of an additional chromogen. However, the quantitative determination of these complex sulfoglycolipids could be achieved using the azure A procedure when these compounds were assayed after peracetylation. Using the modified Kean procedure, the behavior of these sulfoglycolipids in the chloroform–methanol–water partition system was compared.—**Tadano-Aritomi, K., and I. Ishizuka.** Determination of peracetylated sulfoglyco lipids using the azure A method. *J. Lipid Res.* 1983. **24:** 1368–1375.

Supplementary key words monosulfoglycolipid • bis-sulfoglycolipid • seminolipid • acetylation

Sulfogalactosylceramide (S_{M4s}), sulfolactosylceramide (S_{M3}), and seminolipid (S_{M4g}) are well known components of mammalian tissues. Recently, sulfoglycosphingolipids containing *N*-acetylglucosamine (4, 5) and containing sialic acid (6–9) were isolated. We found three *N*-acetylgalactosamine-containing sulfoglycosphingolipids in rat kidney. They were characterized as monosulfogangliotriaosylceramide (S_{M2}) (10, 11), bis-sulfogangliotriaosylceramide (S_{B2}) (12, 13), and bis-sulfogangliotetraosylceramide (S_{B1a}) (14). The number of known sulfoglycolipids has increased, and they constitute a group of glycoconjugates comparable to gangliosides. Thus a simple and accurate method for quantitative determination of many kinds of sulfolipids is required. Although several methods for determination of sulfolipids have been published, most of them are based on the measurement of sulfate liberated by acid hydrolysis (15–17). Kean (18) reported a rapid nonhydrolytic method for the quantitative estimation of sulfatide (S_{M4s}) using a cationic dye, azure A. This method is based on the ability of the sulfatide to form colored azure A complex which is extractable into chloroform–methanol solutions. This method was applied to the estimation of several sulfoglycolipids. In the case of sulfoglycolipids with a longer carbohydrate chain than S_{M3}, however, the intensity of the color was very weak. In the present study, we have demonstrated that these sulfoglycolipids can be successfully estimated by azure A assay after peracetylation. Using the modified Kean procedure, the behaviors of several sulfolipids in chloroform–methanol–water systems (19) were compared.

Abbreviations: S_{M4s}, sulfogalactosylceramide (GalCer-I^3-sulfate); S_{M3}, sulfolactosylceramide (LacCer-II^3-sulfate); S_{M2}, monosulfogangliotriaosylceramide ($GgOse_3Cer$-II^3-sulfate); S_{M1}, monosulfogangliotetraosylceramide ($GgOse_4Cer$-II^3-sulfate); S_{B2}, bis-sulfogangliotriaosylceramide ($GgOse_3Cer$-II^3,III^3-bis-sulfate); S_{B1a}, bis-sulfogangliotetraosylceramide ($GgOse_4Cer$-II^3,IV^3-bis-sulfate); S_{M4g}, seminolipid (Galβ1-3alkylacylGro-I^3-sulfate); lyso-S_{M4g}, lysoseminolipid (Galβ1-3alkylGro-I^3-sulfate). Abbreviations for gangliosides follow the nomenclature system of Svennerholm (1) and those of other lipids follow that of the IUPAC-IUB Commission on Biochemical Nomenclature (2). Abbreviations for sulfoglycolipids follow the modification (3) of the nomenclature system of Svennerholm. TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials

S_{M4s}, S_{M3}, and G_{M3} were prepared from human kidney (20, 21). S_{M4s}, S_{M2}, S_{B2}, and S_{B1a} were isolated from rat kidney as described previously (10–14). S_{M1} was obtained by solvolysis of S_{B1a} and purified by Iatrobeads column chromatography (14). S_{M4g} (22) and lyso-S_{M4g} were prepared from boar testis (23), and converted into K-salts by partition (24, 25). The purity of these sulfoglycolipids was examined by TLC in three solvent systems: chloroform–methanol–0.2% $CaCl_2$ 60:30:6 (v/v), chloroform–methanol–concentrated ammonia–water 60:35:1:7 (v/v), and chloroform–methanol–acetone–acetic acid–water 8:2:4:2:1 (v/v). Sources of standard glycolipids were the same as described previously (11, 13). GalCer was prepared from the white matter of an adult human brain by Dr. K. Ueno according to the method described previously (24). Sphingomyelin from bovine brain (type I) and cardiolipin from beef heart were purchased from Sigma, St. Louis, and Serdary Research Laboratories Inc. London, respectively. Anhydrous pyridine and acetic anhydride of biochemical grade were obtained from Merck, Darmstadt, and Wako Junyaku, Osaka, respectively. Azure A was supplied by Chroma-Gesellschaft, Stuttgart. All organic solvents and other compounds were of analytical quality unless otherwise specified.

Assay procedure

The sample (0.5 to 8 nmol) was pipetted into a screw-cap culture tube and dried in vacuo over phosphorus pentoxide; it was then acetylated (26, 27). Briefly, the dry residue was dissolved in 0.1 ml of pyridine–acetic anhydride 1:1 (v/v). After standing at room temperature for 18 hr in a sealed tube under nitrogen, 1.0 ml of toluene was added to the reaction mixture and the solvents were evaporated under a gentle stream of N_2 at 35°C. One ml of toluene was added and the solvents were again completely evaporated. Acetylated sulfolipids thus obtained were assayed as described by Kean (18) except that the original assay system was scaled down to about one-third. Chloroform–methanol 1:1 (v/v) 1.5 ml, 1.5 ml of 0.05 N H_2SO_4, and 0.3 ml of dye solution were added to each tube. The contents were mixed for 30 sec by a vortex mixer and centrifuged for 10 min at 800 *g*. The absorbance of the lower phase was measured using a JASCO UNIDEC-505 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo) at 635 nm in a micro-absorption cell of 0.5 ml capacity and 10 mm light path. The values are expressed as the mean ± SEM of triplicate determinations. The blank absorbance (without samples) was 0.083 ± 0.006. This value did not change after the acetylation procedure.

For quantitative estimation of each glycolipid, hexose was determined by the anthrone-sulfuric acid method (28) using lactose or galactose as the standard, by the absorption difference between 635 and 720 nm (24). For estimation of S_{M1} and S_{B1a}, equimolar amounts of lactose and galactose were mixed and used as the standard.

Partition procedure

The partition was performed according to the method of Folch, Lees, and Sloane Stanley (19). The sulfolipids (10–30 nmol) were dissolved in 1.6 ml of chloroform–methanol 2:1 (v/v), and 0.25 volume (0.4 ml) of water with or without 0.88% KCl was added, followed by vigorous mixing. After centrifugation for 10 min at 800 *g*, the lower phase was washed twice with theoretical upper phase. Upper aqueous phases and the final lower organic phase were concentrated to dryness in a vacuum desiccator over blue silica gel followed by phosphorus pentoxide, and then acetylated as described above. When the aqueous phase of partition contained KCl, the acetylated upper phase was redissolved in 1.6 ml of chloroform–methanol 2:1 (v/v). Then 0.4 ml of water was added and acetylated sulfoglycolipids were recovered in the lower phase of the partition system. A portion was taken for determination of sulfoglycolipids as described above. The values are the mean of duplicate determinations. In order to determine the recovery of peracetylated bis-sulfoglycolipids in the lower phase, acetylated S_{B2} and S_{B1a} were partitioned in the Folch system with or without KCl. A portion of lower phase was assayed as described above.

The partition was also performed using the system described by Kean (18) except that water was added instead of azure A reagent. To each tube, containing 10 to 20 nmol of sulfoglycolipid, 1.0 ml of chloroform–methanol 1:1 (v/v), 0.2 ml of water, and 1.0 ml of water with or without 0.05 N H_2SO_4 were added. After mixing and centrifugation, the lower phase was brought to slightly basic pH by the addtion of 10 μl of 0.5 M ammonia in methanol. The mixture was concentrated to dryness and assayed for sulfolipid. The solvent composition of the upper phase was made up similar to that of the assay system by the addition of the theoretical upper and lower phases containing azure A. After mixing and centrifugation, the absorbance of the lower phase was measured.

RESULTS

Sulfoglycolipids used in this study were found to be homogeneous by examination with TLC in neutral (**Fig. 1**), basic, and acidic solvent systems. They moved faster

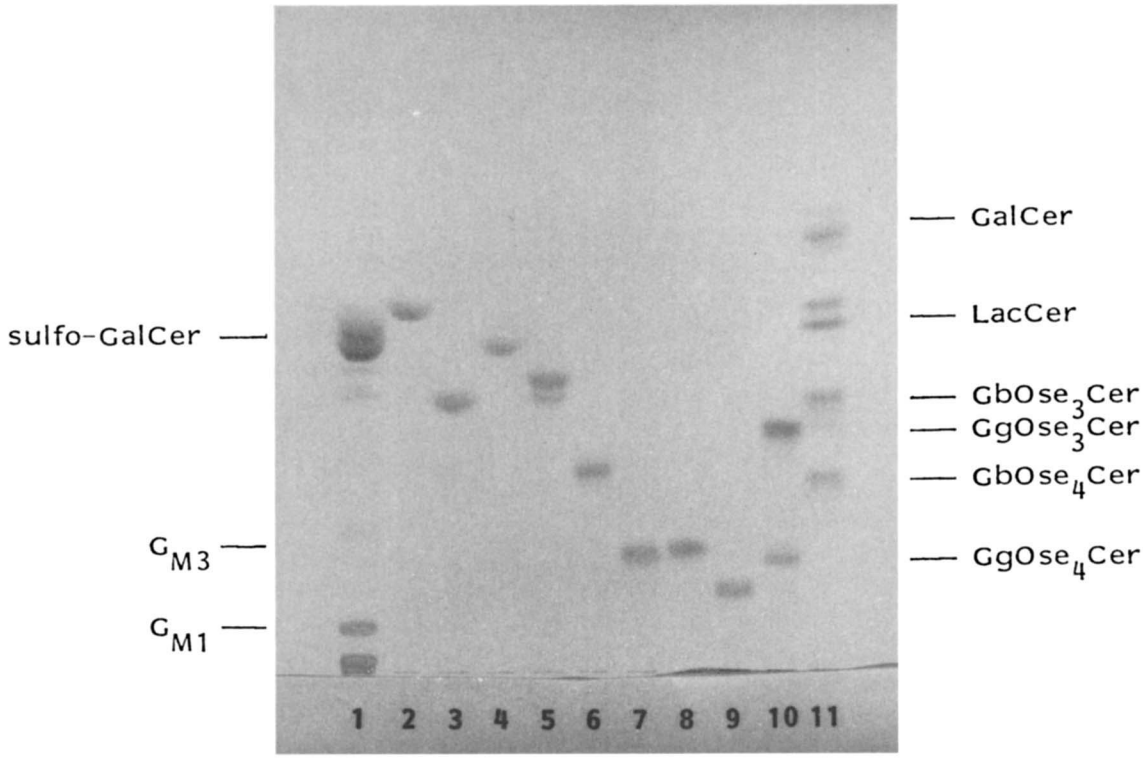

Fig. 1. Thin-layer chromatogram of sulfoglycolipids. The plates were developed with chloroform–methanol–0.2% $CaCl_2$ 60:30:6 (v/v), and stained with orcinol reagent. Lane 1, the mixture of acidic lipids from rat brain; lane 2, S_{M4g}; lane 3, lyso-S_{M4g}; lane 4, S_{M4s}; lane 5, S_{M3}; lane 6, S_{M2}; lane 7, S_{B2}; lane 8, S_{M1}; lane 9, S_{B1a}; lane 10, $GgOse_3Cer$ (upper band) and $GgOse_4Cer$ (lower band); lane 11, GalCer, LacCer, $GbOse_3Cer$, and $GbOse_4Cer$ from top to bottom.

than gangliosides having similar carbohydrate backbone on TLC in all solvent systems tested.

Examination of the spectra between 500 and 750 nm of the azure A complex formed by native S_{M4s}, S_{M3}, S_{M4g}, and lyso-S_{M4g} showed a peak of absorption at 635 nm. This value was slightly different from that described by Kean (647 nm) (18). Acetylation of these sulfoglycolipids did not change the spectra. Native S_{M2} and S_{M1} produced turbidity in the lower phase of the assay mixture and it made a significant contribution to nonspecific absorbancy between 500 and 620 nm (data not shown). In addition to turbidity, the color obtained from native S_{B2} and S_{B1a} was weak and reddish, and had an extra maximum of the absorbance between 550 and 600 nm (**Fig. 2**). This absorbance between 550 and 600 nm was much more intense at higher concentrations of S_{B2} and S_{B1a}, but could not be observed with monosulfoglycolipids. The acetylated S_{M2}, S_{M1}, S_{B2}, and S_{B1a}, however, gave a similar pattern of spectra to S_{M4s}, or other sulfolipids (Fig. 2). Routine analyses were performed at 635 nm.

The color yield of native S_{M4s} was similar to that of acetylated S_{M4s} (**Fig. 3, Table 1**). When acetylation was

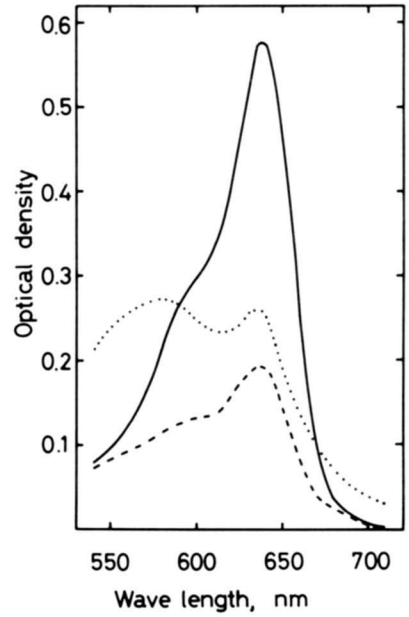

Fig. 2. Absorption spectra of the azure A complex formed by native and acetylated S_{B2}. Native and acetylated S_{B2} were assayed as described in the text. Native S_{B2}, 3.1 (– – –) and 7.5 (· · ·) nmol; acetylated S_{B2}, 3.1 nmol (——).

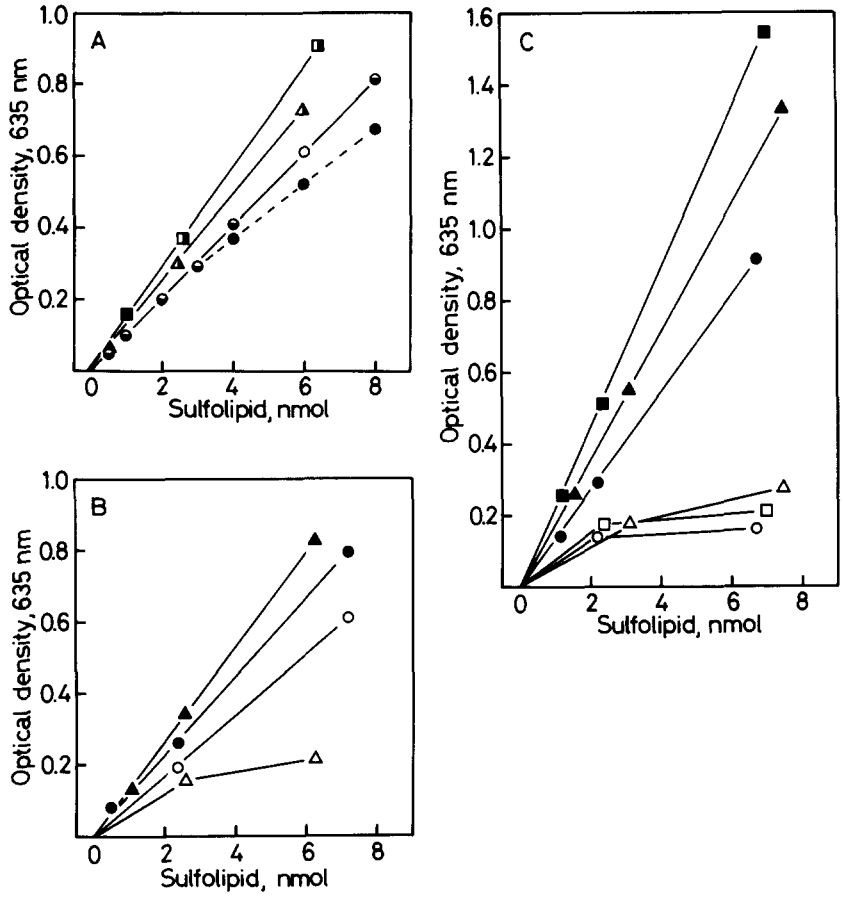

Fig. 3. Proportionality between the absorbance of the color complex formed and the amount of each sulfoglycolipid. Open and closed symbols show native and acetylated sulfoglycolipids, respectively. The half-open-closed symbols indicate the overlapping of both open and closed symbols. Acetylation was performed in 0.1 ml of pyridine–acetic anhydride 1:1 (v/v) at room temperature for 18 hr (——) or at 100°C for 15 min (– – –) as described in the text. A: ○, S_{M4s}; △, S_{M4g}; □, lyso-S_{M4g}. B: ○, S_{M3}; △, S_{M2}. C: ○, S_{M1}; △, S_{B2}; □, S_{B1a}. The values are expressed as the mean of triplicate determinations. The standard errors were less than 5% of the mean values and not shown in the figure.

performed at room temperature, the absorbance of the color was proportional to the amount of S_{M4s} used in the assay over the range from 0.5 to 8.0 nmol. When S_{M4s} was acetylated in the 0.1 ml of reaction mixture at 100°C for 15 min, however, the color was not strictly proportional to the concentration ranges higher than 3 nmol (Fig. 3A). Linearity was obtained if acetylation was performed in 0.6 ml of reaction mixture at 100°C. It might be expected that a part of S_{M4s} was desulfated during acetylation at 100°C with a higher concentration of the compound. Therefore, lower concentrations of the material (less than 30 μM in pyridine–acetic anhydride) was required to avoid desulfation and to get linearity when acetylation was performed at 100°C. For routine analyses, sulfoglycolipids were acetylated at room temperature.

S_{M3} without acetylation reacted as sulfolipid by the azure A procedure but the color yield was 82% compared to that obtained with S_{M4s} (Table 1). The acetylated S_{M3} produced similar yield of color to S_{M4s}; the color was proportional over the range 0.5 to 7.2 nmol used in the assay (Fig. 3B).

The color yields of the native S_{M2} and S_{M1} were 25 and 12%, respectively, of that of S_{M4s} (Fig. 3, B and C). Moreover, accurate determinations could not be achieved because the absorption curve of the complex formed by native S_{M2} and S_{M1} was subject to turbidity as described above. This might have been partly due to the more hydrophilic nature of S_{M2} and S_{M1} as compared to S_{M4s}. The color yields of native S_{B2} and S_{B1a} at 635 nm were less than 25% of that of S_{M4s}. Because of the appearance of an additional color absorbance between 550 and 600 nm (Fig. 2), intensity of the absorbance at 635 nm was not linear to the concentration (Fig. 3C) and accurate amounts of these bis-sulfolipids could not be determined. In contrast, acetylated S_{M2}, S_{M1}, S_{B2},

TABLE 1. Color yields of sulfoglycolipids, other lipids and salts

Compound	Color Yields[a] Native			After Acetylation	
	Absorbance per 100 nmol				
S_{M4s}	10.2	± 0.2	1.00[b]	10.0 ± 0.3	0.99[b]
S_{M3}	8.4	± 0.2	0.82	11.1 ± 0.2	1.09
S_{M2}	2.6	± 0.4[c]	0.25	13.2 ± 0.2	1.29
S_{M1}	1.2	± 0.1[c]	0.12	13.0 ± 0.3	1.29
S_{B2}	2.6	± 0.2[c]	0.25	17.8 ± 0.1	1.76
S_{B1a}	1.9	± 0.2[c]	0.19	21.9 ± 0.2	2.17
S_{M4g}	12.4	± 0.1	1.22	12.1 ± 0.2	1.20
Lyso-S_{M4g}	14.2	± 0.2	1.39	14.3 ± 0.3	1.40
GalCer	<0.1			<0.1	
G_{M3}	1.8	± 0.1	0.18	0.6 ± 0.02	0.06
Sphingomyelin	<0.1			<0.1	
Cardiolipin	3.4	± 0.1	0.33	2.4 ± 0.1	0.23
	Absorbance per 100 μmol				
Potassium chloride	1.10 ± 0.03				
Ammonium acetate	0.08 ± 0.002				

The values are expressed as the mean ± S.E.M. of triplicate determinations.

[a] Absorbance of the colored complexes at 635 nm.

[b] Correcting factor using S_{M4s} as the standard.

[c] Color yields of native S_{M2}, S_{M1}, S_{B2},and S_{B1a} were not linear to the concentration (see the text). These values were calculated from the absorbances with about 7 nmol of compounds.

and S_{B1a} showed 29, 29, 76, and 117% greater molar absorbance, respectively, than S_{M4s}. The intensity was linear up to 8 nmol used in the assay (Fig. 3, B and C).

S_{M4g} had 22% greater molar absorbance than S_{M4s} with or without acetylation (Table 1). Surprisingly, lyso-S_{M4g} gave a 14% higher absorbance than S_{M4g} even when it was not acetylated. This was unexpected because more than 90% of lyso-S_{M4g} was recovered in the upper aqueous phase of chloroform–methanol–water partition system (see below). The color was proportional over the range from 1.0 to 6.4 nmol used in the assay (Fig. 3A).

From these results, it was demonstrated that all sulfoglycolipids used in this study could be assayed by the method of Kean (18) after acetylation, and that the intensities of the colors were significantly different from each other. When S_{M4s} was used as the standard, data from other sulfoglycolipids could be corrected using the following factors: 1.20 and 1.40 for S_{M4g} and lyso-S_{M4g}, respectively, both with and without acetylation; 1.09, 1.29, 1.29, 1.76, and 2.17 for acetylated S_{M3}, S_{M2}, S_{M1}, S_{B2}, and S_{B1a}, respectively (Table 1).

The ability of some lipids other than the sulfolipids to produce color in the azure A assay was investigated (Table 1). Less than 1% of the color intensity was produced by GalCer (5 to 10 nmol) and sphingomyelin (10 to 50 nmol) with or without acetylation. The color yield from native G_{M3}, tested over the range 5 to 10 nmol, was 18.5% of that produced by an equimolar amount of S_{M4s}. Acetylated G_{M3}, over a similar range, however, contributed only 5.8%. Cardiolipin, tested over the range 5 to 50 μg, had 33% of the color yield produced by equimolar amounts of S_{M4s} in agreement with the value reported by Kean (18). After acetylation it was reduced to 23%. It was concluded that the interference of gangliosides and phospholipids was smaller after acetylation.

Using the modified Kean method, behaviors of several sulfoglycolipids in chloroform–methanol–water partition system (11) were compared (**Table 2**). Without KCl in the system, 47.1% of S_{M4s}, and 63.6% of S_{M4g} remained in the lower organic phase. Results with S_{M4s} were in good agreement with those reported by Ishizuka et al. (24) using radioactive S_{M4s}. Only 6.9% of lyso-S_{M4g} was recovered in the lower phase. S_{M3}, S_{M2}, and S_{M1} remained in the lower phase (23.2, 15.6, and 14.0%, respectively). The recoveries of S_{B2} and S_{B1a} in the lower phase were only 5.0 and 1.7% respectively.

When KCl was used in the system, more than 90% of S_{M3} and S_{M2} was recovered in the lower phase after one partition (Table 2). After three partitions, 81.0% of S_{M3} and 73.7% of S_{M2} remained in the lower organic phase. Thirty percent of S_{M1} moved to the upper phase after one partition, and 31.9% was recovered in the lower phase after three partitions. In the case of bis-sulfoglycolipids, more than 60 and 75%, respectively of S_{B2} and S_{B1a} were moved to the upper phase by one partition and less than 7% remained in the lower phase after three partitions, even when KCl was used. Lyso-S_{M4g} was recovered in the upper phase (49.6%) after partitioning once. Only 11.3% of lyso-S_{M4g} appeared in the lower phase after three partitions with 0.88% KCl. On the other hand, it was found that peracetylated bis-sulfoglycolipids were almost completely recovered from the lower organic phase with KCl in the system (Table 2). In the absence of KCl, however, about 15% of acetylated S_{B2} and S_{B1a} were lost in the upper phase.

From the partition experiments, it was concluded that the recovery of sulfoglycolipid in the lower phase was greatly affected by KCl and that the recovery decreased as the number of sugars increased. It was also shown that the behavior of monosulfoglycolipids was different from that of bis-sulfoglycolipids. After three partitions with KCl, the recovery in the lower phase was more than 70% with S_{M3} and S_{M2}. It decreased to about 30% with S_{M1}, and greatly decreased to less than 7% with bis-sulfoglycolipids, S_{B2} and S_{B1a}.

DISCUSSION

Azure A has been applied for the detection of sulfated glycoconjugates on paper (29) and silicic acid-im-

TABLE 2. Partition of sulfoglycolipids into two phases

Compound	KCl or H_2SO_4 in Upper Phase	Up 1[a]	Up 2	Up 3	Lp	Total
Folch system						
S_{M4s} (rat)	−	51.3			47.1	98.4
(human)	−	54.5			43.3	97.8
S_{M3}	−	79.8			23.2	103.0
	+	5.2	4.6	4.0	81.0	94.9
S_{M2}	−	80.0			15.6	95.6
	+	6.6	6.3	5.5	73.7	92.2
S_{M1}	−	83.9			14.0	97.9
	+	30.0	22.2	18.6	31.9	102.7
S_{B2}	−	95.0			5.0	100.0
	+	62.2	25.2	10.4	7.0	104.8
S_{B1a}	−	97.4			1.7	99.1
	+	77.2	13.7	4.7	2.0	97.6
S_{M4g}	−	38.3			63.6	102.7
Lyso-S_{M4g}	−	94.1			6.9	101.2
	+	49.6	27.9	12.7	11.3	101.7
Peracetylated S_{B2}	−				85.3	
	+				99.5	
Peracetylated S_{B1a}	−				85.4	
	+				98.6	
Kean system						
S_{M4s}	−	25.6			63.7	89.3
	+	14.7			74.6	89.3
Lyso-S_{M4g}	−	86.6			2.6	89.2
	+	79.1			9.8	88.9

The partition was performed using 1.6 ml of chloroform–methanol 2:1 (v/v) and 0.4 ml of water (0.88% KCl) (Folch system), or 1.0 ml of chloroform–methanol 1:1 (v/v), 0.2 ml of water, and 1.0 ml of water (0.05 N H_2SO_4) (Kean system) as described in the text. The values are the mean of duplicate determinations.

[a] Up, upper aqueous phase; Lp, lower organic phase.

pregnated paper (16). Kean (18) successfully applied this pigment for the quantitative estimation of sulfated amphiphiles (3). He studied steroid sulfates and S_{M4s} but not other sulfoglycolipids, and mentioned in his report that dihexose sulfatides are probably reactive (18). In this study, several kinds of monosulfoglycolipids as well as bis-sulfoglycolipids have been found to behave as sulfolipids by the method of Kean (18). However, it was shown that unmodified monosulfoglycolipids with longer carbohydrate chains than S_{M3} and bis-sulfoglycolipids could not be determined quantitatively because of low color yields, turbidity, and the appearance of an additional chromogen. Pieringer et al. (30) determined the quantity of S_{M4g} by the method of Kean using S_{M4s} as the standard. Slomiany et al. (4, 5) reported that sulfolactoneotriaosylceramide and sulfolactoneotetraosylceramide reacted as sulfolipids in the azure A procedure but quantitative data were not shown in his studies. Our study showed that even the color yields of native S_{M4s} and S_{M4g} differed significantly from each other, probably due to the difference of solubilities of both dye-sulfoglycolipid complexes.

The color production by potassium chloride and ammonium acetate in the assay procedure was examined (Table 1). Although it has been reported that the color yields from these salts on a molar basis were quite low and that no color was observed in the lower phase of the lipid wash (18), the strong color was obtained with the upper phase. In the modified Kean method, salts could be removed from the assay system by partition after acetylation without loss of acetylated sulfolipids (Table 2). This method permits the accurate determination of sulfolipids even in the presence of large amounts of salts.

The method described in this paper is more sensitive (lower limit, about 0.5 nmol) and simpler than others such as those based on liberation of sulfate by acid hydrolysis (lower limit, 10 to 100 nmol) (31). In addition, the methods based on acid hydrolysis are subject to error if nonlipid sulfate is present, and require the use of specialized glassware. This study showed clearly that the modified Kean procedure is applicable to the accurate determination of purified or partially purified samples of the various sulfoglycolipids. However, application of the method to the crude tissue samples seems to be difficult. Table 1 shows that the various sulfolipids had different color yields. It also indicates that G_{M3} (and probably other gangliosides) and cardiolipin produced 6 and 24%, respectively, of the color intensity produced by S_{M4s}. Therefore, the accurate determination could

not be obtained when tissue samples contain several kinds of sulfoglycolipids and/or relatively large amounts of gangliosides and/or cardiolipin. In some cases, however, such as fractions from DEAE-Sephadex column, which contain only one kind of sulfoglycolipid and relatively small amounts of gangliosides or cardiolipin, this method is applicable, because large amounts of salts can be removed from the assay system.

Using the modified Kean method, behaviors of several sulfoglycolipids in the partitioning system of Folch et al. (19) could be compared. It was reported that gangliosides G_{M2} and G_{M1} appeared about 95 and 100%, respectively, in the upper phase after partition with water (32, 33). With 0.88% KCl in the system, about 30 and 100%, respectively, of G_{M2} and G_{M1} were partitioned in the upper phase (33, 34). Our results showed that about 80 and 84%, respectively, of S_{M2} and S_{M1} were transferred into the upper phase after partition with water. In the presence of 0.88% KCl, S_{M2} and S_{M1} were recovered about 93 and 70%, respectively, from the lower organic phase, indicating that larger amounts of sulfoglycolipids were recovered in the lower organic phase as compared to gangliosides containing a similar carbohydrate backbone and a similar number of negative charges. Lingwood, Hay, and Schachter (35) used a partition method without KCl for the purification of ^{35}S-labeled sulfolipids from rat organs. Table 2 shows that even in the case with S_{M4g} and S_{M4s}, about 40 to 50% was removed to the upper phase, and more than 80% of sulfoglycolipids with a longer carbohydrate chain than S_{M3} would be lost in the upper phase. It was reported that gangliosides could be partitioned in a chloroform-rich phase by addition of other lipids or salts (33, 34). Although the behavior of sulfoglycolipids in the partition system must be changed by the presence of other lipids or salts similar to the case with gangliosides, our data provides useful information concerning the purification and isolation steps of sulfoglycolipids.

From the behavior of lyso-S_{M4g} in the Folch et al. (19) partition system, lyso-S_{M4g} seems more hydrophilic than S_{M1}. However, the color yield of lyso-S_{M4g} in the azure A procedure was higher than that of S_{M4g}. In order to know the recovery of lyso-S_{M4g} in the lower phase of the Kean assay system, the partition of native lyso-S_{M4g} was performed without azure A in the system. Table 2 shows that only 2.6 and 9.8% were recovered in the lower organic phase with or without H_2SO_4, respectively. Therefore, the higher yield of the color with lyso-S_{M4g} in the presence of azure A may be due to the formation of the complex with azure A, which is more lipophilic.

There have been several reports about the interaction of gangliosides with various cationic compounds including serotonin (36–38) and basic proteins (39, 40). Recently, an assay for gangliosides based on specific ganglioside-serotonin binding was reported (41). However, the values were markedly affected by the presence of various types of gangliosides or sulfatide. It is suggested that the interaction of sulfoglycolipids with the cationic dye, azure A, is similar in nature to that of gangliosides with various cationic compounds. Azure A was shown to interact specifically with negative charges of sulfate groups rather than sialic acid or phosphate (18). It is obvious that various degrees of specificities exist in these interactions.

It has been suggested that the presence of two sulfate groups significantly increases the solubility of bile acid in urine. However, sulfated glycolipids of mammalian kidney have been suggested to be the component of renal tubular epithelial cells (42). The biological significance of these highly acidic amphiphiles might rather be the ion-exchanger on the cell surface similar to sulfated glycoproteins and glycosaminoglycans.

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