Micellar properties of glycosphingolipids in aqueous media

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Abstract The aggregation properties of neutral glycosphingolipids and gangliosides were investigated by ultracentrifugal sedimentation and gel permeation chromatography. Initially, glycosphingolipids in buffer formed high molecular aggregates. On standing, the glycosphingolipids produced smaller and stable micelles in equilibrium with their monomers at the critical micellar concentration (cmc). The cmc's of monohexaosyl- to tetrahexaosylceramides were on the order of 10^{-8} to 10^{-7} M. In contrast, values of 10^{-8} M for monosialogangliosides and 10^{-6}–10^{-5} M for di- and trisialogangliosides were found. From estimations of hydrodynamic radii, Svedberg coefficients, and partial specific volumes, relative masses of glycosphingolipid micelles were calculated to be in the range of 300 to 1000 for the neutral glycosphingolipids and 100 to 350 for the gangliosides.—**Ulrich-Bott, B., and H. Wiegandt.** Micellar properties of glycosphingolipids in aqueous media. *J. Lipid Res.* 1984; **25:** 1233–1245.

Supplementary key words gangliosides • micelles • critical micellar concentration

Knowledge of multimer aggregation behavior of polar lipids in aqueous media is important in understanding properties of biological membranes. This may appear particularly relevant with those membrane lipids that by themselves are freely water-soluble, as is the case with the higher gangliosides (for review, see 1). The gangliosides with a comparably smaller carbohydrate moiety, e.g., gangliosides $G_{gal}1$, $G_{lac}1$, and $G_{tri}1$, are less water-soluble and resemble more closely the neutral glycosphingolipids. At low concentrations, corresponding to their respective solubilities, all glycosphingolipids can be expected to form micellar aggregates in aqueous media. For the gangliosides, it was shown in several earlier and more recent reports that between 200 and 350 monomers exist as micelles of an oblate ellipsoid shape with a hydrodynamic radius of some 60 Å (2, 3). However, the published data on the critical concentration of micelle formation show wide discrepancies, ranging in an early report from 10^{-4} M (4) to values as low as 10^{-9} M, found in more recent investigations (5, 6). For an easily water-soluble ganglioside, the latter data appeared surprisingly low and suggested the possibility of physicochemical properties specific for these sialo-glycosphingolipids.

In the present investigation, the aggregation behavior of human brain gangliosides was systematically reinvestigated and compared to that of other neutral glycosphingolipids.

EXPERIMENTAL PROCEDURES

High performance thin-layer plates, Silica Gel 60 and Silica Gel H were from Merck, Darmstadt, FRG; Dowex 50 WX2 was from Serva, Heidelberg, FRG; DEAE-Sephadex A25 and Sepharose 4B were products of Pharmacia, Uppsala, Sweden. Scintillation cocktail 808 E was obtained from Riedel de Haën AG, Seelze-Hannover, FRG; sodium boro-[^{3}H]hydride (273 mCi/mmol) was from Amersham-Buchler GmbH, Braunschweig, FRG. All other reagents were of the highest purity available; the organic solvents were freshly distilled before use.

Preparation of glycosphingolipids

Glycosphingolipids were isolated from human tissues, except ganglioside $G_{lac}2$ (pig kidney), a gift of Dr. Ineo Ishizuka, Tokyo.

Galactosylceramide and the gangliosides were prepared from a chloroform–methanol extract of human brain. After alkaline saponification of the extract, extensive dialysis against water and precipitation with acetone,

Abbreviations: cmc, critical micellar concentration; GSL, glycosphingolipid; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; TLC, thin-layer chromatography; Gal-Cer, galactosylceramide; Lac-Cer, lactosylceramide; Gb_3Cer, globotriaosylceramide; Gg_3Cer, gangliotriaosylceramide; Gg_4Cer, gangliotetraosylceramide; $G_{gal}1$, NeuAcα2-3Galβ1-1Cer; $G_{lac}1$, II^{3}NeuAc-LacCer; $G_{lac}2$, II^{3}NeuAc$_2$-LacCer; $G_{tri}1$, II^{3}NeuAc-Gg_3Cer; $G_{tri}2$, II^{3}NeuAc$_2$-Gg_3Cer; $G_{tet}1$, II^{3}NeuAc-Gg_4Cer; $G_{tet}2a$, IV^{3}NeuAc-, II^{3}NeuAc-Gg_4Cer; $G_{tet}2b$, II^{3}NeuAc$_2$-Gg_4Cer; $G_{tet}3b$, IV^{3}NeuAc-, II^{3}NeuAc$_2$-Gg_4Cer; $G_{tet}4b$, IV^{3}NeuAc$_2$-, II^{3}NeuAc$_2$-Gg_4Cer; $G_{tri}1$-ol, II^{3}N-acetylneuraminulosyl-Gg_3Cer (N-acetylneuraminulose = 5-N-acetamido-3,5-dideoxy-D-glycero-β-D-galactononulose).

the nonsaponifiable lipids were separated into neutral and acidic fractions by DEAE-Sephadex A 25 ion-exchange chromatography, according to Ledeen, Yu, and Eng (7). Galactosylceramide was purified from the neutral fraction by repeated recrystallization from hot methanol by adding activated charcoal.

Single ganglioside components were isolated from the crude fraction of the acidic lipids and purified by repeated high pressure liquid chromatography.

Neutral GSL's were isolated from human spleen (LacCer) and human erythrocytes (Gb_3Cer) or prepared by partial hydrolysis of gangliosides (Gg_3Cer, Gg_4Cer) as described before (8). All neutral GSL's were finally purified by HPLC.

High performance liquid chromatography

Neutral GSL's were separated on Silica Gel H; column size 0.4 cm diameter, 20 cm length. Elution was with solvents according to Watanabe and Arao (9); the HPLC-column was equilibrated with solvent I: 2-propanol–hexane 55:45 (v/v). GSL, 2–3 mg, dissolved in 100–200 μl of 2-propanol–hexane–water 55:40:5 was applied

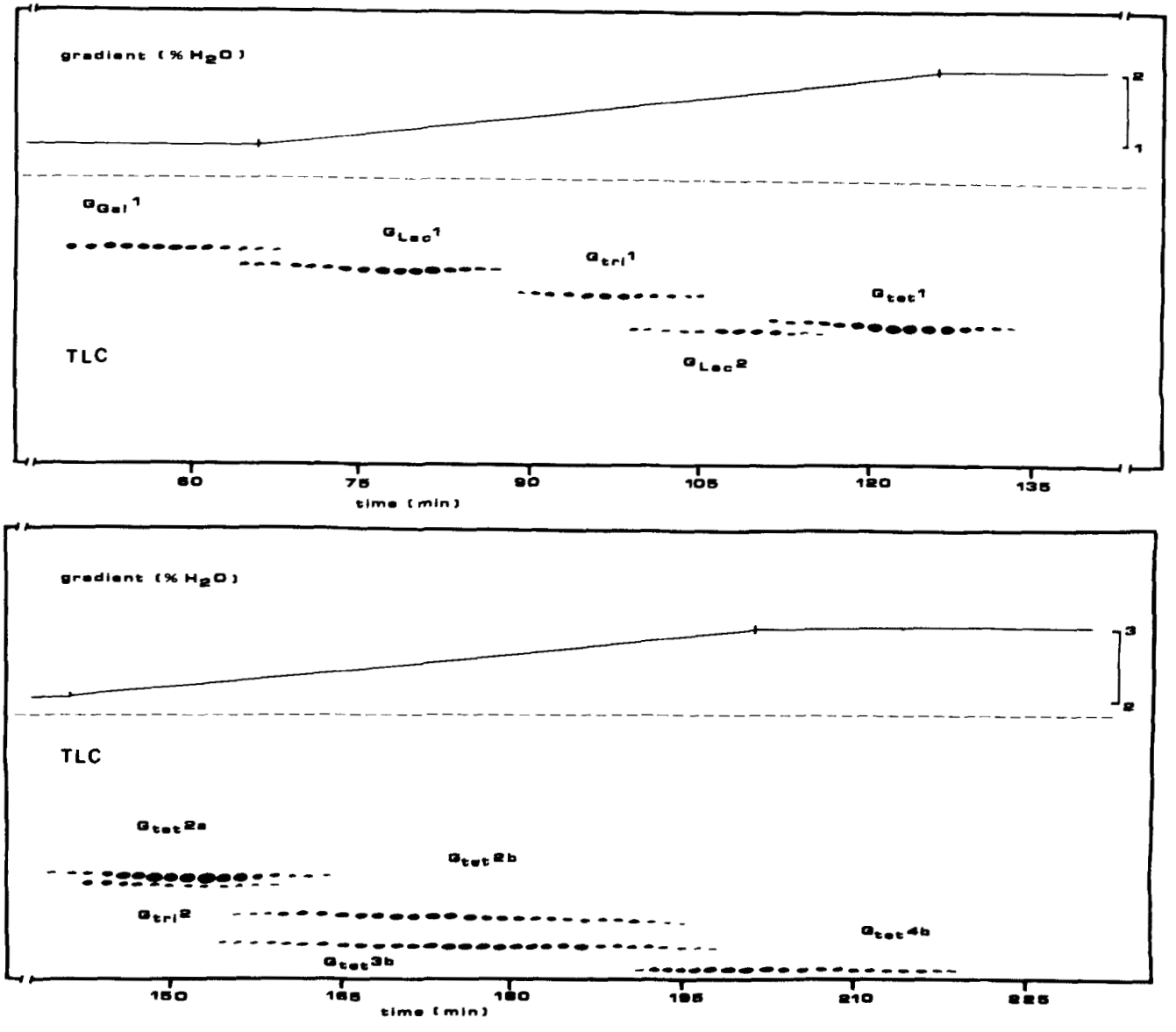

Fig. 1. Typical elution pattern of mixed brain gangliosides by HPLC. Thin-layer chromatographic behavior of ganglioside fractions obtained after separation by HPLC (column 0.4 cm diameter; 20 cm length; packing Silica Gel H; elution program (chloroform–methanol–hexane–water (v/v)): 35 ml of 35:20:45:0; 30 ml of 35:20:44:1; 60 ml of a linear gradient of 35:20:44:1 to 35:20:43:2; 15 ml of 35:20:43:2; 60 ml of a linear gradient of 35:20:43:2 to 38:27:32:3; 30 ml of 38:27:32:3. Solvent system for TLC was chloroform–methanol–water (0.3% $CaCl_2$) = 60:35:8 (v/v). The gangliosides were visualized with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde/HCl).

to the column. After rinsing with 60 ml (1 ml/min) of solvent I, the GSL's were eluted with a 40-ml (1 ml/min) linear gradient of 2-propanol–hexane–water 55:44:1 (solvent II) to 55:35:10 (solvent III) (elution of Gal-Cer, Lac-Cer, Gb_3Cer, Gg_3Cer), followed by isocratic elution with 20 ml (1 ml/min) of propanol–hexane–water 55:33:12 (v/v) (elution of Gg_4-Cer).

Gangliosides were separated by HPLC on Silica Gel H with chloroform–methanol–hexane–water. The column, described above was equilibrated with chloroform–methanol–hexane 35:20:45 (v/v). Two to five mg of gangliosides in 100–300 μl chloroform–methanol–water 10:10:1 (v/v) were applied and elution was performed as described in the legend to **Fig. 1.** Corresponding fractions, as monitored by TLC, were combined, brought to dryness, and rechromatographed, if purity was insufficient.

Radiolabeling of glycosphingolipids

All GSL's were tritiated in the ceramide moiety using boro-[^{3}H]hydride in the presence of palladium catalyst (8). After labeling, tritiated GSL's were separated from radioactive impurities by HPLC using the same solvent systems as described above. Specific activities of the products were established by estimation of the neutral GSL's according to the method of Ohsawa, Nakane-Hikichi, and Nagai (10), by HPTLC with defined GSL's as standards (gift fom Dr. Takako Ohsawa, Tokyo). Gangliosides were estimated colorimetrically with orcinol-sulfuric acid (11). The specific radioactivities (dpm/nmol) were: 47,600 for Gal-[^{3}H]Cer; 26,700 for Lac-[^{3}H]Cer; 37,000 for Gb_3-[^{3}H]Cer; 153,000 for Gg_3-[^{3}H]Cer; 45,900 for Gg_4-[^{3}H]Cer; 538,500 for [^{3}H]G_{gal}1; 272,800 for [^{3}H]G_{lac}1; 250,800 for [^{3}H]G_{lac}2; 153,000 for [^{3}H]G_{tri}1; 307,700 for [^{3}H]G_{tet}1; 18,700 for [^{3}H]G_{tet}2a; 24,300 for [^{3}H]G_{tet}2b; 17,500 dpm for [^{3}H]G_{tet}3b. Since [^{3}H]G_{tri}1-ol was prepared from ganglioside G_{tri}1, it was assumed to have the same specific radioactivity.

Preparation of II^{3}Neuraminulosyl-gangliotriaosyl-[^{3}H]ceramide

Ganglioside [^{3}H]G_{tri}1 (540 μg, dried over P_2O_5) was dissolved in dry methanol (1 ml) and 2 mg of Dowex 50 (H^+-form, dried in vacuo over P_2O_5) was added. The mixture was stirred at room temperature for 4 days. Ester formation was monitored by TLC (solvent: chloroform–methanol–water 65:25:4, (v/v); methylester R_f of 0.70, visualized by radiofluoroautography). After removal and extensive washings of the ion exchange resin with methanol, the washings and the sample solution were combined and reduced in volume (1 ml). Then the methylester was reduced to the II^{3}N-acetyl-neuraminulosyl-gangliotriaosyl-[^{3}H]ceramide by dropwise addition to a solution of precooled sodium borohydride in 0.2 M borate buffer (pH 8.0) at 4°C, with continuous stirring (12 hr, 4°C). After dialysis against water at 4°C, the sample was lyophilized. The product was purified by HPLC. II^{3}N-acetyl-neuraminulosyl-gangliotriaosyl-[^{3}H]ceramide migrated on HPTLC-silica gel plates, running solvent chloroform–methanol–water 65:25:4 (v/v) with R_f with 0.36, i.e., faster than the parent ganglioside G_{tri}1 (R_f was 0.08, visualized by radiofluoroautography). The product was characterized by hydrolysis under very mild conditions (Dowex 50, H^+-form, 20 min, 40°C) that yielded gangliotriaosyl-[^{3}H]ceramide as identified by TLC and radiofluoroautography with the authentic standard GSL and N-acetyl-neuraminulose (5-N-acetamido-3,5-dideoxy-D-glycero-β-D-galactononulose). The latter deoxy-nonulosamine does not yield the violet color with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde/HCl) that is typical for sialic acids. With diphenylamine, however, the N-acetylnonulosamine gives the blue-violet color reaction, well suited for detection on silica gel or cellulose thin-layer chromatograms.

Diphenylamine-reaction on chromatograms

Solution A: diphenylamine in ethanol (1% w/v). Solution B: orthophosphoric acid (density of 1.71 g/ml) in n-butanol (10% w/v). The chromatograms were sprayed with a 1:1 (v/v) mixture of solution A and B. This then was heated for 3–15 min at 90°C. N-acetyl-neuraminulose or oligosaccharides containing this sugar appeared blue-violet after a few minutes, whereas free or bound sialic acid became pink-violet only after some 15 min.

Radiofluoroautography

HPTLC-separated [^{3}H]GSL's were visualized as described previously (8).

Preparation of aqueous GSL solutions

[^{3}H]GSL's were dried from solution in organic solvent and redissolved in aqueous buffer (Tris-HCl), 100 mM; EDTA, 1 mM; pH 7.4). After brief sonification, the sample solutions were generally stored 1 to 7 days at 4°C before centrifugation or gel filtration chromatography.

Gel permeation chromatography

Sepharose 4B (column: 1 × 50 cm) was used for molecular sieving chromatography at room temperature with Tris-HCl (100 mM), NaCl (140 mM), EDTA (1 mM), pH 7.4, buffer as eluent. The sample was applied to the column in 200 μl of solution and fractions of 800 μl were collected. Radioactivity of each fraction was determined by liquid scintillation counting. Molecular hydrodynamic radii of GSL's were calculated from elu-

tion volumes by the method of Ackers (1967), using blue-dextran (v_0, exclusion volume), phenol-red (v_t, total volume), bovine serum albumin (M_r, 68,000), aldolase (M_r, 150,000), and catalase (M_r, 222,000) as standards.

Band sedimentation

Sedimentation was performed on continuous sucrose gradients (5–20% sucrose), buffered with Tris-HCl (100 mM) in the presence of EDTA (1 mM) at pH 7.4. The sample (200 μl) in the same buffer was carefully layered on top of a preformed gradient (10 ml). Centrifugation was at 4°C or 20°C and 35,000 rpm (rotor SW 41 Ti, Beckman). After completion of the run, fractions of 0.5 ml were collected beginning from the bottom of the tubes. Radioactivity of each fraction was determined by liquid scintillation counting.

Isopycnic sedimentation

Caesium chloride, 2 g (for mono-, di-, and trihexaosyl-ceramides) or 4 g (for tetrahexaosyl-ceramides or gangliosides) was dissolved in 10 ml of buffer (Tris-HCl, 100 mM; EDTA, 1 mM; pH 7.4). After addition of 100 μl of a solution of GSL in the same caesium chloride solvent, centrifugations were performed for 72 hr at 20°C and 35,000 rpm (rotor SW 41 Ti, Beckman). At the end of the run, fractions of 0.5 ml were collected and GSL's were detected by scintillation counting of an aliquot of each fraction. The density of fractions containing GSL's was determined by their refractive index.

Calculations

Apparent sedimentation constants (S_{app}) were determined according to Svedberg and Peterson (12). Svedberg constants ($S_{20,w}$) were calculated according to Martin and Ames (13).

The sucrose density of each fraction was obtained by refractometry and the viscosity of the sucrose solutions was calculated by interpolation from data of Landolt-Börnstein (14) and Bates (15).

The relative micellar masses (M_r) of glycosphingolipids were calculated according to Tanford et al. (16) from the value of hydrodynamic radii (R_s) as determined by gel permeation chromatography and Svedberg constants.

RESULTS

Separation and purification of GSL's

All GSL's that were investigated were purified by HPLC before use. Gangliosides were first separated into mono-, di-, tri-, and tetrasialospecies on DEAE Sephadex (17). Single components were then isolated by HPLC on Silica Gel H with chloroform–methanol–hexane–water as solvent (Fig. 1). After radiolabeling by tritiation, all [^{3}H]GSL's were purified by HPLC again (**Fig. 2**).

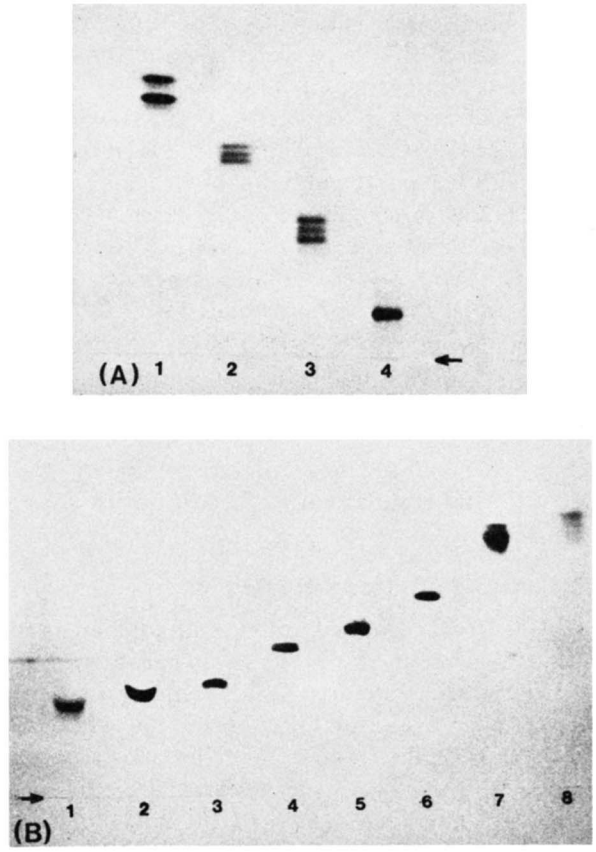

Fig. 2. High performance thin-layer chromatograms of the neutral [^{3}H]GSL's and [^{3}H]gangliosides for use in this study after purification on HPLC. A, Neutral GSL's: 1) Gal-Cer, 2) Lac-Cer (human spleen); 3) Gb_3-Cer (human erythrocyte); 4) Gg_4-Cer (human brain) (solvent system: chloroform–methanol–water 65:25:4 (v/v)); B, Gangliosides: from human brain: 1) $G_{tet}3b$; 2) $G_{tet}2b$; 3) $G_{tet}2a$; 4) $G_{lac}2$; 5) $G_{tet}1$; 6) $G_{tri}1$; 7) $G_{lac}1$; 8) $G_{gal}1$; from pig kidney (solvent system: chloroform–methanol–water 60:35:3 (v/v).

Critical micellar concentration

GSL's in aqueous solution, including gangliosides, form stable aggregates, probably micelles, that can be separated from monomers' by gel permeation chromatography or by ultracentrifugal sedimentation. Below their assumed cmc, exclusively monomeric GSL was observed forming a nonsedimenting fraction during ultracentrifugation (**Fig. 3**). Upon gel permeation chromatography, GSL-monomers are eluted close to the total volume (**Fig. 4**).

Above certain concentrations, besides monomers, higher aggregates are seen that sediment in the ultra-

[1] Even though no proof for true monomers is available, GSL's behaving as such will henceforth be called monomeric.

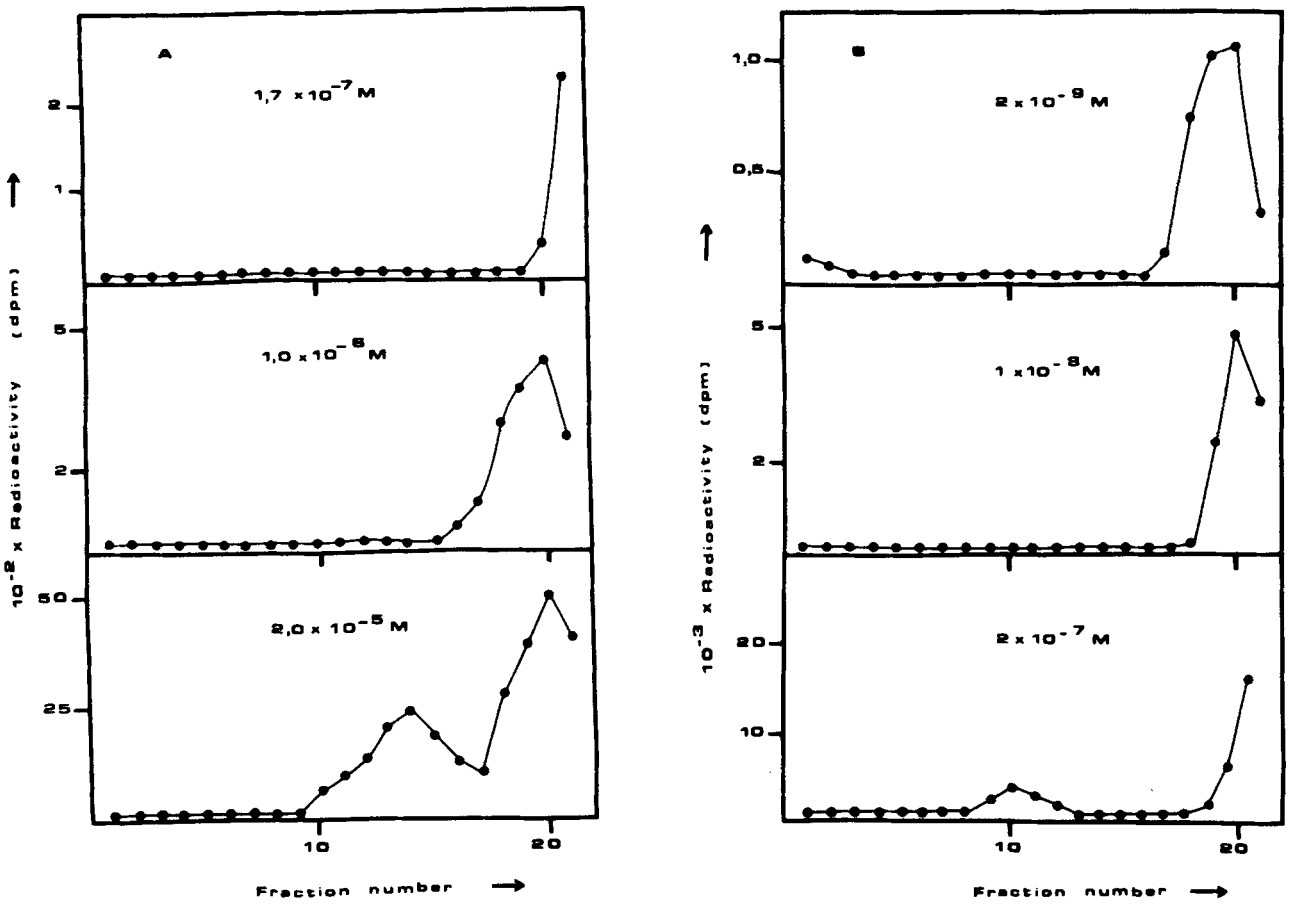

Fig. 3. Ultracentrifugal sedimentation of ganglioside [^{3}H]G_{tet}3b (A) and the neutral GSL Lac-[^{3}H]Cer (B) at different concentrations. Aliquots of the glycolipids stored in organic solvent at −20°C were evaporated to dryness and dissolved in 100 mM Tris-HCl and 1 mM EDTA, pH 7.4, with short sonification. After standing at room temperature for 1 hr (G_{tet}3b) to 6 days (Lac-Cer), 100 μl of solution was put on top of a preformed gradient (5–20% sucrose in the above buffer) and centrifuged for 17 hr (A) or 15 hr (B) at 20°C (35,000 rpm, rotor SW 41 Ti, Beckman). Fractions of 500 μl were collected beginning from the bottom of the tubes and counted for radioactivity.

centrifuge, or else elute upon gel chromatography earlier than the monomers.

Using these two methods, neutral GSL's as well as gangliosides are shown to behave similarly, forming micelles in aqueous solution. The concentrations, however, that are necessary to obtain higher molecular aggregates varied according to the glycolipid species. These concentrations, estimated at 20°C, are given in **Table 1.** The cmc's were calculated from the concentration of the respective GSL's in the nonsedimenting fraction (ultracentrifugation) or found at the position of the monomer peak (gel permeation chromatography), at the point of initial micellar formation. Both methods lead to comparable results.

In order to confirm that the radioactivity detected at the position of monomers and micelles indeed belongs to the unaltered original GSL, both fractions were analyzed by thin-layer chromatography. All the radioactivity in the fractions of GSL monomers as well as micelles was thus identified to originate solely from the respective undegraded compounds, as shown, e.g., for Gal-[^{3}H]Cer in **Fig. 5.**

Using this approach, the calculated cmc of GSL's was found to be in the range between 10^{-5} and 10^{-8} M. For gangliosides with sialo-oligosaccharide moieties up to a relative molecular weight of 1000, similar cmc's in the range of 10^{-8} M were calculated. In contrast, the corresponding "asialo-gangliosides" showed cmc's between one-half to one order of magnitude higher than the parent gangliosides (Table 1). This implicates sialic acid in determining the low cmc's of certain gangliosides.

In order to assess more precisely the role of the negatively charged sialic acid carboxyl group influencing the cmc's, the gangliosides G_{tet}1, G_{tri}1, and G_{lac}1 were investigated below their pK-values, at pH 1.5.[2] At this

[2] Under the conditions chosen, i.e., pH 1.5, 12 hr incubation at 20°C, no degradation of the investigated gangliosides [^{3}H]G_{tet}1, [^{3}H]G_{tri}1, and [^{3}H]G_{lac}1 was detected by TLC and radiofluoroautography.

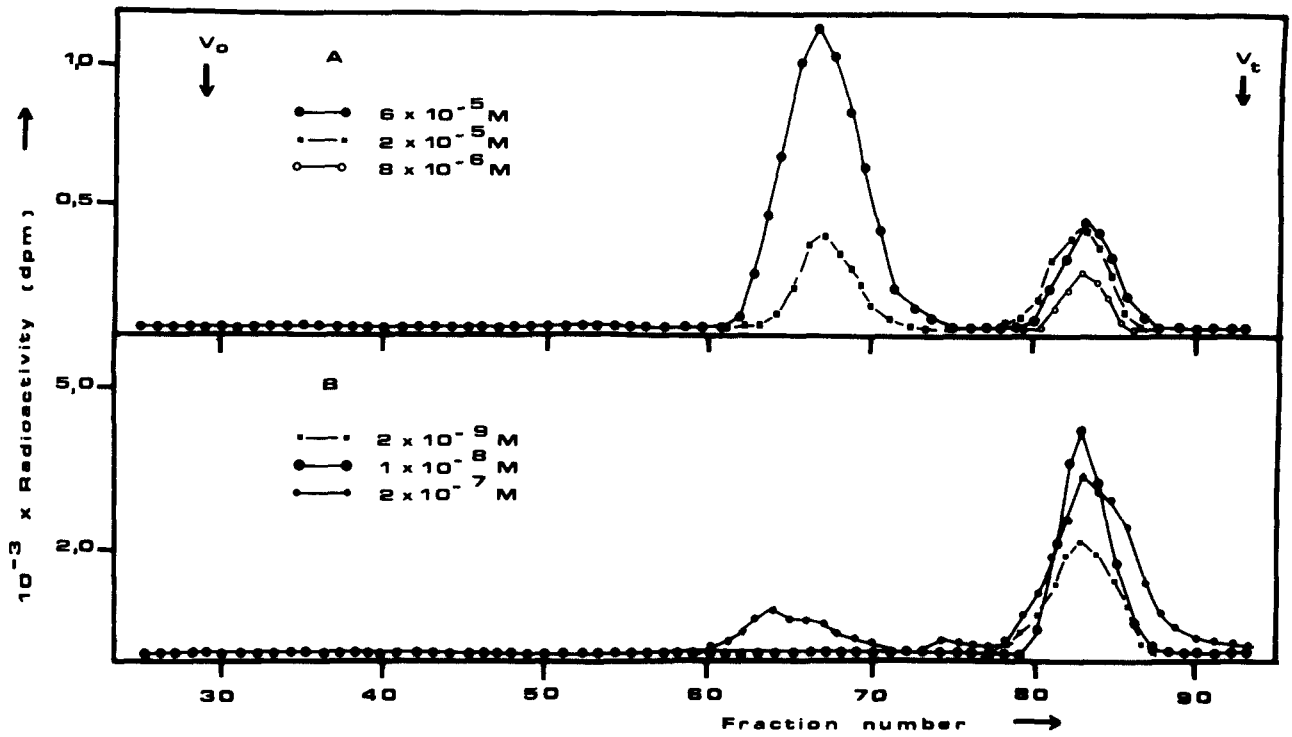

Fig. 4. Gel filtration chromatography profile of ganglioside [^{3}H]G_{tet}3b (A) and neutral GSL Lac-[^{3}H]Cer (B) at different concentrations. Sample solutions were prepared as described in Fig. 3. For each separation, 200 μl was applied to a Sepharose 4B column (1 × 50 cm) equilibrated with 100 mM Tris-HCl, 1 mM EDTA, and 140 mM NaCl, pH 7.4. The same buffer was used for sample elution (10 ml/hr) at room temperature. Fractions of 800 μl were collected and aliquots were counted for radioactivity.

pH the cmc's increased one-half to one order of magnitude as compared with those found at pH 7.4 (Table 1).

Furthermore, reduction of the sialic acid of ganglioside G_{tri}1 at the carboxyl group to the corresponding N-acetyl-neuraminulopyranosyl residue raised the cmc by one order of magnitude (Table 1, **Fig. 6**).

As compared to the parent monosialo-ganglioside G_{tet}1, the disialo-gangliosides G_{tet}2a and G_{tet}2b have higher cmc's by two, and the trisialo-ganglioside G_{tet}3b even three orders of magnitude. In this case the higher content of sialic acid raises the cmc by one to two orders of magnitude for each additional neuraminic acid residue (Table 1).

TABLE 1. Values for the cmc (M) of glycosphingolipids[a]

GSL	pH 1.5[b]	pH 7.4[b]
Gal-Cer		$5.0 \pm 0.5 \times 10^{-8}$ (16)
Lac-Cer		$1.5 \pm 0.5 \times 10^{-7}$ (27)
Gb_3-Cer		$4.0 \pm 0.5 \times 10^{-7}$ (8)
Gg_3-Cer		$1.0 \pm 0.5 \times 10^{-7}$ (6)
Gg_4-Cer		$1.5 \pm 0.5 \times 10^{-7}$ (13)
G_{tri}1-ol		$5.0 \pm 2.0 \times 10^{-7}$ (6)
G_{gal}1		$2.0 \pm 0.5 \times 10^{-8}$ (8)
G_{lac}1	$5.0 \pm 0.5 \times 10^{-8}$ (7)	$2.0 \pm 0.5 \times 10^{-8}$ (23)
G_{lac}2		$2.0 \pm 0.5 \times 10^{-8}$ (8)
G_{tri}1	$1.0 \pm 0.5 \times 10^{-7}$ (14)	$5.0 \pm 2.0 \times 10^{-8}$ (30)
G_{tet}1	$2.5 \pm 0.6 \times 10^{-7}$ (10)	$2.0 \pm 1.0 \times 10^{-8}$ (36)
G_{tet}2a		$2.0 \pm 1.0 \times 10^{-6}$ (12)
G_{tet}2b		$1.0 \pm 0.5 \times 10^{-6}$ (7)
G_{tet}3b		$1.0 \pm 0.5 \times 10^{-5}$ (11)

[a] Determined at 20°C.
[b] The number of experiments performed is in parentheses.

Characterization of glycosphingolipid aggregates

The formation of aqueous GSL solutions with more uniform, stable aggregates may depend on the structure of the oligosacharide moiety and the divalent cation content of the GSL, its concentration, pH of the medium, and the time of incubation.

Shortly after suspension of a GSL in aqueous media, in addition to monomers and micelles, comparably higher aggregates can be observed by ultracentrifugal sedimentation as well as by gel permeation chromatography. These aggregates disintegrate into micelles and monomers at equilibrium within minutes to several days, following the solubilities of the GSL's (**Fig. 7** and **Fig. 8**).

Indeed, with the less water-soluble GSL, mono- and dihexaosylceramide, even after prolonged incubation, rearrangement of higher molecular structures is still incomplete. In such a case "insoluble material" was removed by prior centrifugation for 1 hr at 100,000 *g*.

In contrast, the highly water-soluble gangliosides

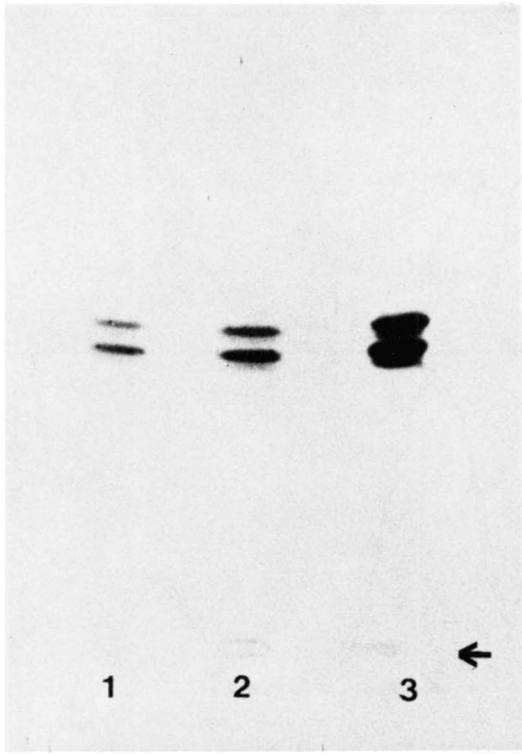

Fig. 5. Thin-layer chromatography of Gal-[^{3}H]Cer before and after ultracentrifugal sedimentation. Gal-[^{3}H]Cer, micellar and monomeric peak, were separately pooled after ultracentrifugation. Both fractions were dialyzed (4 days, 4°C), lyophilized, and re-chromatographed. (TLC solvent system was chloroform–methanol–water 65:25:4 (v/v)). The spots were visualized by radioautography. 1) Monomeric Gal-[^{3}H]Cer; 2) micellar Gal-[^{3}H]Cer; 3) original Gal-[^{3}H]Cer.

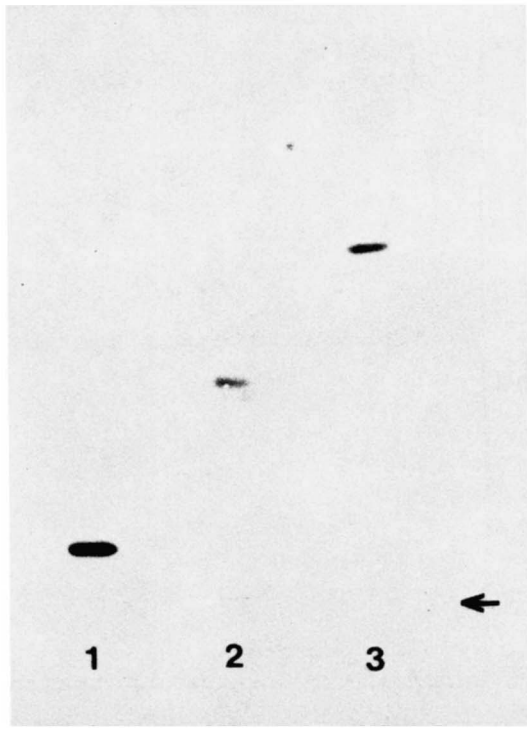

Fig. 6. Thin-layer chromatography of ganglioside [^{3}H]G_{tri}1 (1), [^{3}H]G_{tri}1-ol (2), and Gg_3-[^{3}H]Cer (3). Solvent system for TLC was chloroform–methanol–water 65:25:4 (v/v). Visualization was by radiofluoroautography.

G_{tet}2a, G_{tet}2b, and G_{tet}3b showed an immediate micellization after dissolution in buffer (Figs. 7 and 8). The size of the micelles as reflected by the micellar sedimentation constant depends on the concentration of the ganglioside. An increase in ganglioside concentration was paralleled by a lowering in the micellar sedimentation rate as shown, e.g., for ganglioside G_{tri}1 in **Fig. 9.**

In the case of gangliotetraosylceramide, two kinds of micelles were observed, sedimenting in the ultracentrifuge at different rates. At a concentration well above the cmc, both populations of micelles exist in approximately equal amounts. Closer to the cmc, however, the faster sedimenting species is preferentially diminished, until at 2–5 × 10^{-6} M, only the slower micelles are seen (**Fig. 10, Table 2**).

Similar to what is observed of the critical micellar concentration, the sedimentation behavior of gangliosides also is dependent on the hydrogen ion concentration. At lower pH values the ganglioside micelles have higher apparent sedimentation constants (**Table 3**).

In order to obtain some indication of the stability of ganglioside aggregates, pH 7.4-micelles were centrifuged

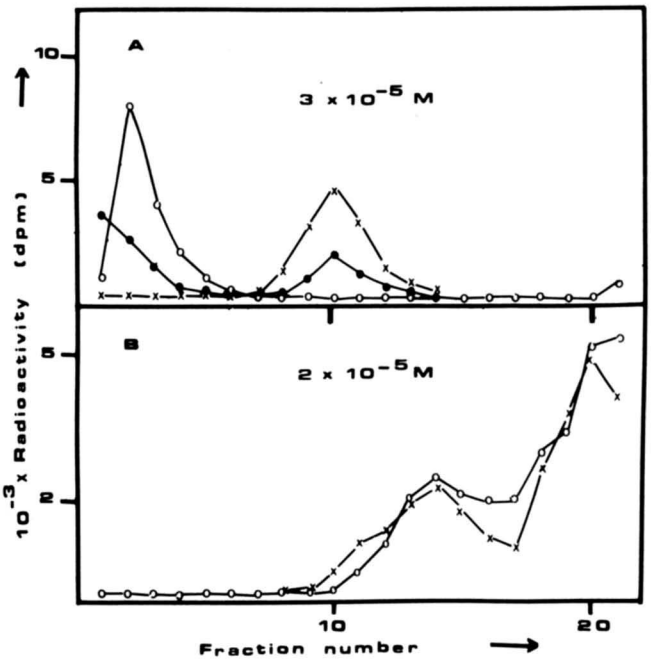

Fig. 7. Ultracentrifugal sedimentation of ganglioside [^{3}H]G_{lac}1 (A) and [^{3}H]G_{tet}3b (B) at different times after solubilization in aqueous buffer. Conditions as given in Fig. 3. Centrifugation time for ganglioside [^{3}H]G_{lac}1 was 15 hr and for [^{3}H]G_{tet}3b was 17 hr. A, (○ —— ○) 1 hr; (● —— ●) 1 day; (x —— x) 6 days; B, (○ —— ○) 1 hr; (x —— x) 3 days.

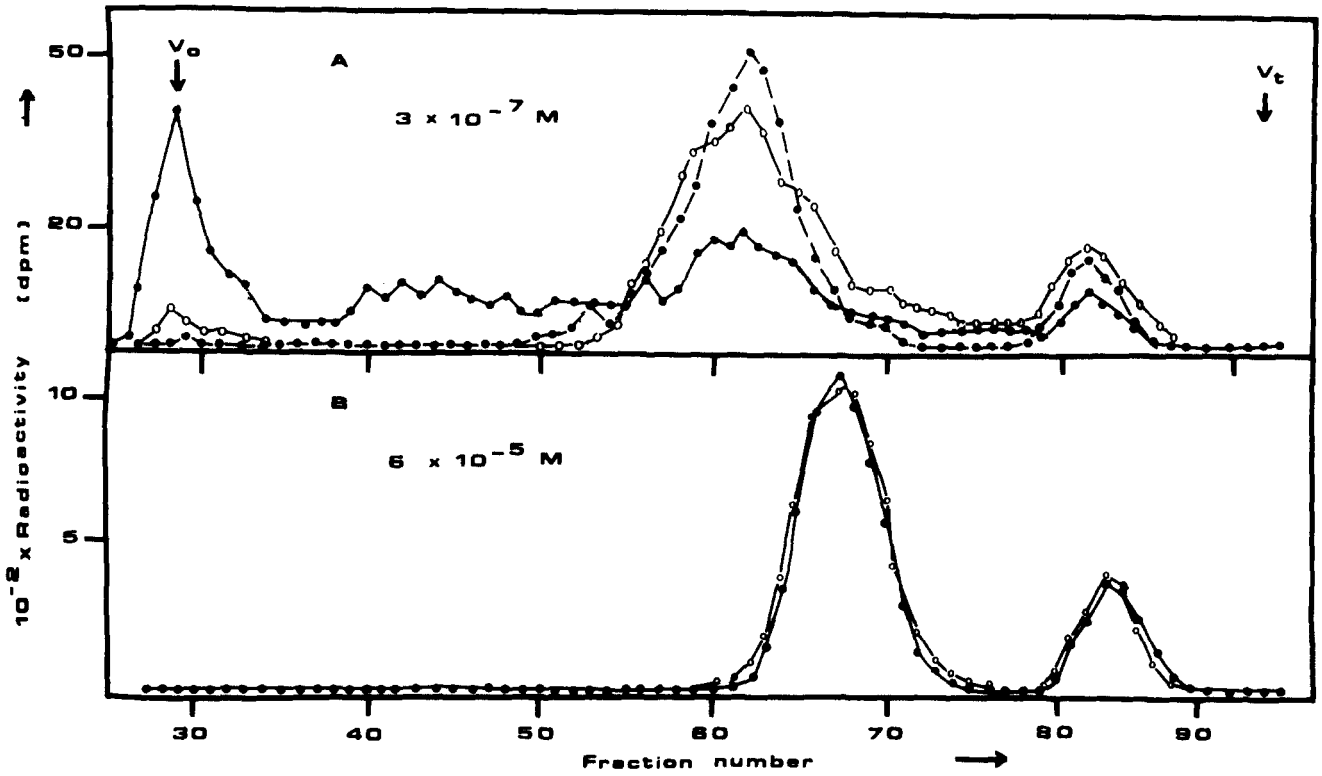

Fig. 8. Gel permeation chromatography of ganglioside [^{3}H]G_{lac}1 (A) and [^{3}H]G_{tet}3b (B) at different times after solubilization in aqueous buffer. Conditions of chromatography as given in Fig. 4. A, (● —— ●) 2 hr; (○ —— ○) 2 days; (■ —— ■) 6 days; B, (● —— ●) 10 min; (○ —— ○) 3 days.

for 15 hr at 20°C on a pH 1.5 continuous sucrose gradient. The monosialogangliosides G_{lac}1, G_{tri}1, and G_{tet}1, which were investigated under such conditions, each sediments as a micellar peak with two maxima, whose sedimentation velocities are the same as found for pure pH 7.4- and pH 1.5-micelles (**Fig. 11**). In the presence of 10 mM Mg^{2+}, no change of the sedimentation

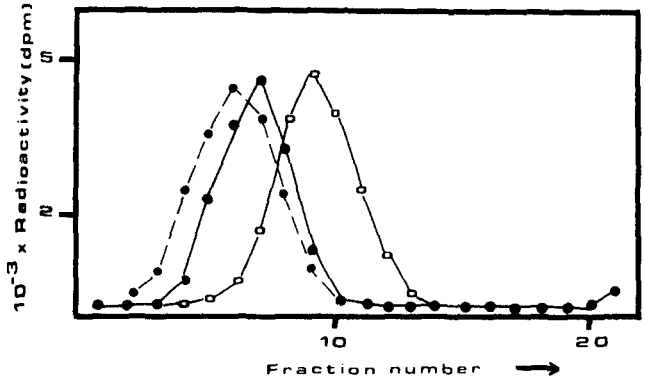

Fig. 9. Sedimentation behavior of ganglioside [^{3}H]G_{tri}1 at different concentrations above the cmc. Conditions as given in Fig. 3, centrifugation time 20 hr. Sample concentrations were adjusted by addition of cold ganglioside to the tritium-labeled compound; (○ —— ○) 3.5×10^{-4} M; (● —— ●) 3.5×10^{-5} M; (● - - - ●) 1×10^{-7} M.

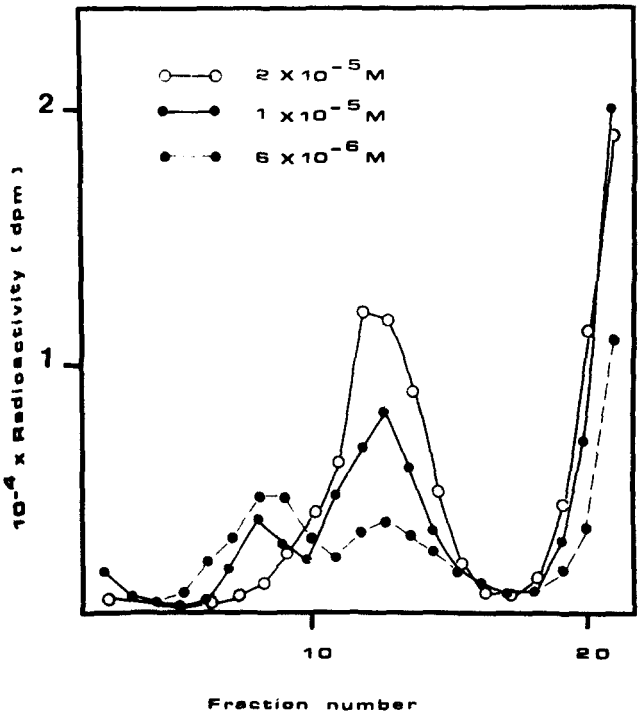

Fig. 10. Sedimentation behavior of glycosphingolipid Gg_4-[^{3}H]Cer at different concentrations above the cmc. Conditions as given in Fig. 3; centrifugation time 15 hr.

TABLE 2. Physico-chemical properties of GSL micelles at 10^{-6} M concentration

	S_{app} [s] (±0.2)	$S_{20,w}$ [s] (±0.3)	R_s [Å] (±2)	M_r [dalton] ($\times 10^{-3}$)	Micellar Number
Neutral GSL's					
Gal-Cer	4.9	11.1	60	1049 ± 74	1496 ± 89
Lac-Cer	6.4	12.1	60	729 ± 43	822 ± 48
Gb_3-Cer	5.7	9.3	n.d.	n.d.	n.d.
Gg_3-Cer	5.4	7.5	62	356 ± 26	327 ± 24
Gg_4-Cer	4.5 (6.4)	6.4 (9.9)	n.d.	n.d.	n.d.
G_{tri}1-ol	5.3	7.8	60	279 ± 20	205 ± 15
Gangliodies					
G_{gal}1	2.7	4.4	n.d.	n.d.	n.d.
G_{lac}1	4.2	7.8	60	322 ± 24	273 ± 20
G_{lac}2	4.5	6.6	60	281 ± 23	191 ± 16
G_{tri}1	4.8	7.6	60	281 ± 21	204 ± 14
G_{tet}1	6.4	9.7	60	337 ± 22	218 ± 14
G_{tet}2a	4.2	6.2	57	181 ± 15	99 ± 8
G_{tet}2b	3.9	5.6	57	160 ± 14	87 ± 8
G_{tet}3b (10^{-5} M)	3.6	4.5	55	113 ± 12	53 ± 6
Standard Proteins					
Bovine serum albumin	2.7	4.1 (4.6[a])	(35[b])	(68[a])	
Aldolase	4.8	7.3 (7.4[a])	(46[b])	(150[a])	
Catalase	9.9	11.9 (11.2[a])	(52[b])	(222[a])	

[a] Lehninger, A. L. 1979. Biochemie, 2nd Edition. Verlag Chemie, Weinheim, New York, p. 140.
[b] Tanford et al. (16).

behavior of gangliosides G_{lac}1, G_{tri}1, and G_{tet}1 was observed. However, 10 mM Ca^{2+} caused a shift to higher apparent sedimentation values similar to that of lowering the pH.

Isopycnic sedimentation

For estimation of apparent partial specific volumes, the radiolabeled GSL's were sedimented in caesium chloride gradients at pH 7.4. The partial specific volumes found were in fair agreement with values approximated by calculation from the relative molecular proportions of their lipophilic and hydrophilic moieties (**Table 4**).

Sedimentation constants

Micelles of various GSL's sedimented in the ultracentrifuge with different velocities; their sedimentation properties were compared to those of proteins of known molecular mass at 4°C and 20°C (Table 2, **Fig. 12**).

The apparent sedimentation constants that were determined for neutral and acidic GSL's are given in Table 2. From these values and those for partial specific volumes as obtained by isopycnic sedimentation, Svedberg coefficients were calculated. The data show a decrease in sedimentation rate of neutral GSL's with relative increase in the size of the carbohydrate moiety. In a different fashion, the gangliosides show an increase in the apparent sedimentation rate in the series of the monosialo-compounds from G_{gal}1, G_{lac}1, G_{tri}1 to G_{tet}1; the latter ganglioside showed the highest value of all sialoglycosphingolipids investigated.

Further substitution of ganglioside G_{tet}1 with additional sialic acid residues, however, lowers the rate of sedimentation (Table 2).

Gel permeation chromatography

In general, the results of gel chromatography of GSL's were in agreement with those obtained by ultracentrifugal sedimentation. By chromatography on Se-

TABLE 3. Apparent sedimentation constants of gangliosides: dependence on the H^+-concentration or the presence of divalent cations

Ganglioside	pH 1.5[a]	pH 7.4[b]	pH 9.0[c]	10 mM Mg^{2+} [d]	10 mM Ca^{2+} [d]
G_{lac}1	5.1 ± 0.3	4.2 ± 0.2	3.6 ± 0.3	4.2 ± 0.2	5.1 ± 0.2
G_{tri}1	5.7 ± 0.3	4.8 ± 0.2	3.9 ± 0.3	4.8 ± 0.2	5.7 ± 0.2
G_{tet}1	7.2 ± 0.3	6.4 ± 0.2	5.1 ± 0.3	6.4 ± 0.2	7.2 ± 0.2

[a] One hundred mM Glycine-HCl-buffer, 1 mM EDTA.
[b] One hundred mM Tris-HCl-buffer, 1 mM EDTA.
[c] One hundred mM Tris-HCl-buffer, 1 mM EDTA.
[d] One hundred mM Tris-HCl-buffer, pH 7.4.

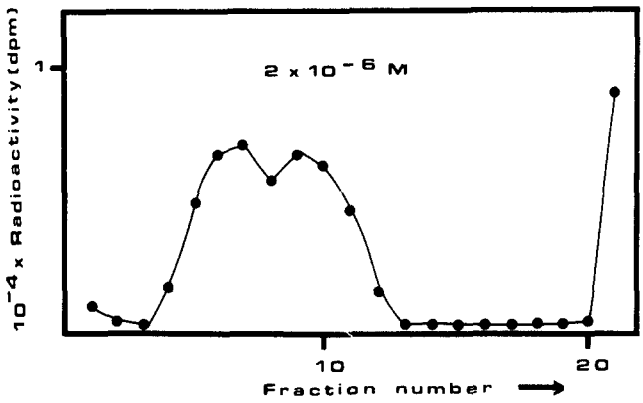

Fig. 11. Sedimentation behavior of ganglioside [^{3}H]G_{tri}1 pH 7.4-micelles on a pH 1.5 sucrose gradient. The ganglioside was dissolved in 100 μl of buffer at pH 7.4 (Tris-HCl, 100 mM and EDTA, 1 mM), kept 1 day at room temperature, and was overlayered on a linear continuous sucrose gradient (5–20% sucrose in 100 mM Glycin-HCl and 1 mM EDTA, pH 1.5). Centrifugation conditions as given in Fig. 3, with centrifugation time of 15 hr.

pharose 4B, GSL monomers could be separated from micelles of intermediate size and from higher aggregates. Monomers were retarded close to the total volume of the chromatography column. GSL micelles eluted as symmetric peaks at positions where optimal linearity of dependence of elution volume and the hydrodynamic radius could be expected.

The Stoke's hydrodynamic radii of glycolipid micelles were calculated from their elution velocities using gel permeation chromatography on calibrated Sepharose 4B columns. All GSL's had Stoke's radii between 55 to 60 Å (Table 2).

Relative micellar masses

Using the values of the Stoke's radii obtained by gel permeation chromatography in conjunction with Svedberg coefficients, the relative micellar masses were calculated (Table 2). The highest M_r of about 1,000,000 with some 1,000 monomers per micelle was calculated for galactosylceramide from brain. Ganglioside micelles had relative micellar masses of approximately 100,000–350,000 corresponding to micellar numbers of some 50–270 monomers.

DISCUSSION

The aggregation properties of gangliosides in aqueous solution have in the past been determined by various indirect techniques, including electric conductivity measurements (4, 18), the absorbancy of the triiodide ion (2), and light scattering (19). With these methods, comparable cmc values for the monosialo-ganglioside G_{tet}1 were found in the range of 10^{-6} M. For the higher sialylated mammalian brain gangliosides G_{tet}2 and G_{tet}3, values between 10^{-5} M and 10^{-4} M were estimated (2). Further evidence for a physico-chemical transition of ganglioside aggregation in this range was the markedly enhanced hydrolysis of the gangliosides G_{tri}1 and G_{tet}1 by *Clostridium perfringens* sialidase below concentrations of 10^{-5} M (20–22).

More direct observations of ganglioside micelles upon sedimentation or gel permeation chromatography, however, revealed that gangliosides still existed in the form of aggregates at concentrations in the order of 10^{-8} M–10^{-9} M, thereby casting doubt on the validity of the previously assumed cmc's (5, 23).

Determination of the cmc of highly purified brain gangliosides in this study indeed confirmed the latter results of an unexpectedly low cmc for these lipids. For monosialo-gangliosides, values in the range of 2–5 $\times 10^{-8}$ M; for disialo-gangliosides G_{tet}2a and G_{tet}2b 10^{-6} M, and for the trisialo-ganglioside G_{tet}3b 10^{-5} M were estimated. Neutral GSL's had cmc's within 5×10^{-8} M–5×10^{-7} M (Table 1).

Experiments performed with aqueous GSL-solutions, equilibrated and centrifuged at 20°C or 4°C, yielded comparable results indicating an unexpected temperature independence.

TABLE 4. Partial specific volumes ($\bar{v}$, ml/g) of neutral and acidic [^{3}H]GSL micelles at 10^{-6} M

	Found	Calculated[a] C_{18}[b]	Calculated[a] C_{24}[b]	Origin
Neutral GSL's				
Gal-Cer	0.928	0.988	1.006	brain, man
Lac-Cer	0.887	0.895	0.916	spleen, man
Gb_3-Cer	0.870	0.840	0.861	erythrocytes, man
Gg_3-Cer	0.852	0.832	0.847	brain, man
Gg_4-Cer	0.814	0.797	0.814	brain, man
G_{tri}1-ol	0.810	0.780	0.797	G_{tri}1
Gangliosides				
G_{gal}1	0.864	0.850	0.871	brain, man
G_{lac}1	0.835	0.810	0.830	brain, man
G_{lac}2	0.840	0.767	0.784	kidney, pig
G_{tri}1	0.816	0.778	0.794	brain, man
G_{tet}1	0.804	0.759	0.775	brain, man
G_{tet}2a	0.779	0.735	0.749	brain, man
G_{tet}2b	0.774	0.735	0.749	brain, man
G_{tet}3b	0.752	0.718	0.731	brain, man

[a] Calculated according to the equation

$$\bar{v}_c = \frac{\text{mol}}{[100 \text{ mol lipid} \times \bar{v} \text{ (stearic acid)}]} + \frac{\text{mol}}{[100 \text{ mol carbohydrate} \times \bar{v} \text{ (sucrose)}]}$$

[b] C_{18}, values for sphing-4-enine/stearic acid; C_{24}, sphing-4-enine/lignoceric acid.

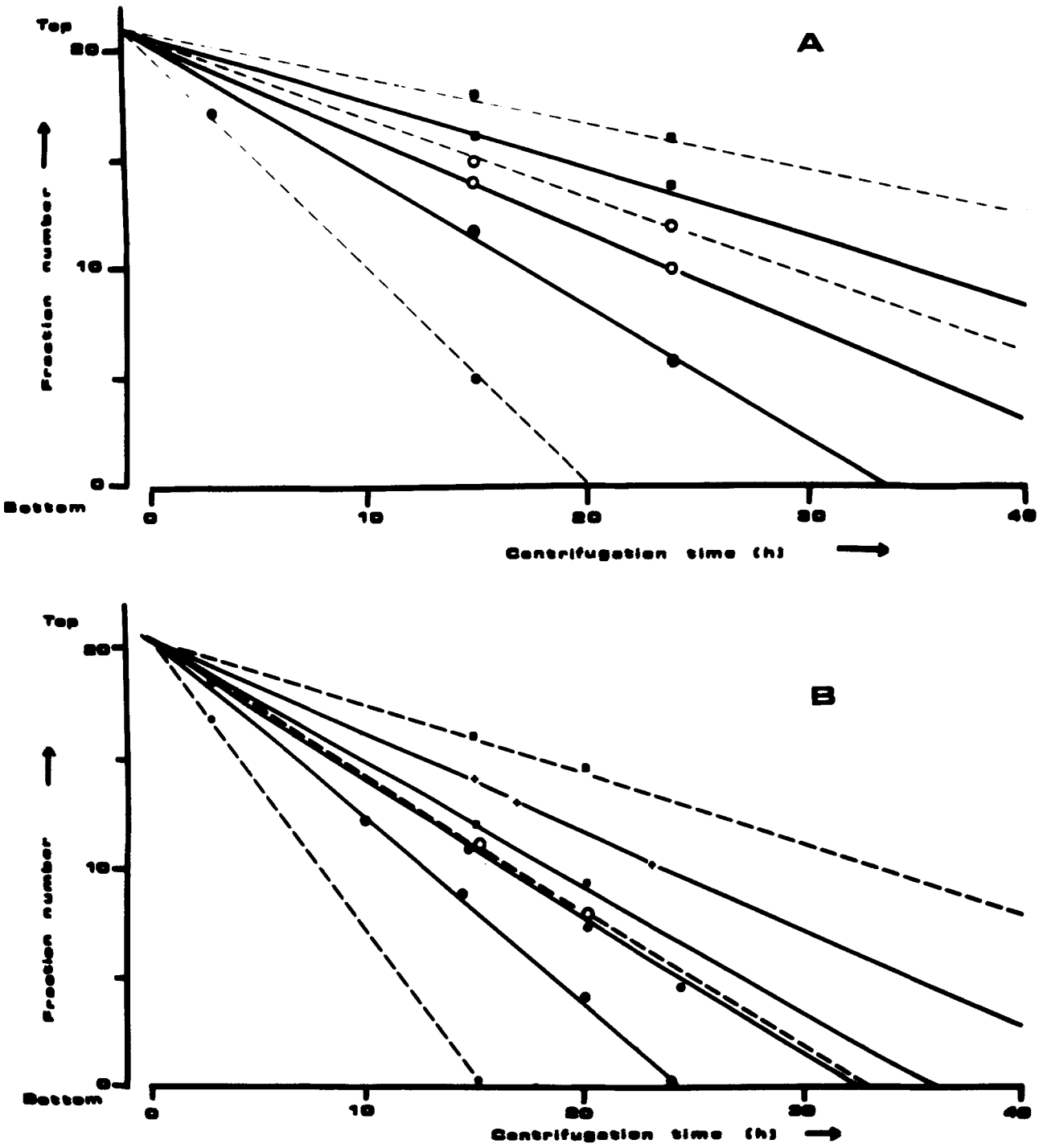

Fig. 12. Comparison of the sedimentation behavior of various ganglioside micelles with standard proteins at 4°C (A) and 20°C (B). Sedimentation conditions as given in Fig. 3. [^{3}H]G_{lac}1 (■ —— ■); [^{3}H]G_{tri}1 (○ —— ○); [^{3}H]G_{tet}1 (● —— ●); [^{3}H]G_{tet}3 (x —— x); bovine serum albumin (M_r 68,000) (■ – – – ■); aldolase (M_r) 150,000) (○ – – – ○); catalase (M_r 222,000) (● – – – ●).

The methods used in this study for the determination of the cmc, the relative molecular mass, and size were sedimentation by ultracentrifugation and gel permeation chromatography. For meaningful evaluation of these non-equilibrium mass transport techniques, it is essential that the rate of micellar disintegration and interconversion should be slow with respect to the relaxation times of sedimentation and gel filtration.

Earlier investigations had already shown that GSL-micelles are very stable structures. Thus, mixing aqueous

solutions containing different gangliosides over long time periods, the original GSL-species could be separated in their micellar form by polyacrylamide gel electrophoresis (24). Essentially no formation of mixed ganglioside micelles was observed during the duration of the experiments made in the present study. The longevity of ganglioside micelles was also shown by equilibrium dialysis (5). Furthermore, disintegration of GSL-micelles should give rise to an observable trailing during sedimentation as well as during gel filtration. However, no such trailing was seen to any significant extent. The longevity of GSL-micelles therefore allows the separation of monomers and micelles within the time frame of the experiments performed. It may, therefore, be possible that GSL-micelles may exist for short time periods even below their cmc. This observation was confirmed by experiments where GSL-micelles separated by ultracentrifugation were immediately centrifuged again under the same conditions as the first run. The monomer concentration after the second centrifugation was found to be much lower than the expected cmc, seen with the first separation procedure (data now shown). An additional indication for the stability of GSL-micelles is the finding that micelles formed at pH 7.4 did not disintegrate completely, even after centrifugation for 15 hr in a sucrose gradient at pH 1.5 (Fig. 11).

When GSL's were brought from the solid state into solution at a final concentration above the cmc, especially with the less water-soluble GSL's, initially higher molecular aggregates were observed. With time, a transition to smaller structures occurs—micelles in equilibrium with monomers. This transition appeared to be comparatively rapid, i.e., less than 1 hr for the readily water-soluble GSL-species, the di- and trisialo-gangliosides. However, the monosialo-gangliosides and the neutral GSL's showed relaxation times for the disintegration of the higher aggregates to "normal" micelles that allowed their observation by ultracentrifugation and gel permeation chromatography. The higher aggregates formed peaks separated from micelles with extensive trailing in between the two (Fig. 8). With the exception of gangliotetraosyl-ceramide, which even after extensive equilibration showed two stable populations of micelles of different size, all estimations of cmc's were done using equilibrated GSL solutions at concentrations where no higher aggregates were present.

The method of Martin and Ames (13), for the determination of the sedimentation behavior of macromolecules in sucrose gradients as established for proteins, was used for lipids with comparably much higher partial specific volumes. All GSL's including galactosylceramide moved at characteristic linear rates during centrifugation, and Svedberg constants determined at different temperature of centrifugation (Fig. 12) were within limits of error identical for a given compound (the latter not shown).

All findings with the ultracentrifuge could be confirmed by parallel investigations by gel permeation chromatography.

There was an obvious dependence of the micellar size on the GSL concentration. With increasing concentration there was a decrease in the apparent sedimentation rate of the micelles. Such concentration-dependent behavior was particularly conspicuous with gangliotetraosylceramide. This GSL formed micelles of two different size populations, one of which, the slower sedimenting species, was favored at higher total GSL concentrations. This phenomenon may perhaps be explained by a destabilization of GSL micelles by the greater frequency of collisions that will occur at higher concentrations resulting in a decrease in micellar size.

For comparison of various GSL's, the Svedberg constants were determined at the same concentration, i.e., 10^{-6} M. For ganglioside $G_{tet}1$,a $S_{20,w}$ of 9.7 was calculated, a value which agrees with earlier estimates ((5), $S_{20,w}$: 10.0;(6),$S_{20,w}$: 10.0). Data for other gangliosides had not previously been determined.

Similar to what was seen with the cmc, the presence of sialic acid greatly influenced the size of the micelles as reflected in their Svedberg coefficients. Gangliosides with only one sialic acid residue had increasing Svedberg constants with longer neutral carbohydrate chains. In contrast, different neutral GSL's generally showed decreasing Svedberg constants relative to a growing carbohydrate chain.

The particular influence of the sialic acid on ganglioside aggregation became apparent when the electric charge of the carboxyl group was altered. Thus, a lowering of the pH, chemical reduction of the carboxyl group to a primary alcohol, or presence of calcium all showed a similar influence, i.e., an increase in the cmc's of the gangliosides.

In agreement with the Svedberg constants, the hydrodynamic radii of the higher sialylated gangliosides (as estimated from the behaviour during gel filtration) decreased with increasing sialic acid content. The value of 60 Å for $G_{tet}1$ is the same as that found by Tettamanti et al. (25) by light scattering methods.

The relative molecular masses of gangliosides were calculated from the Stoke's radii, the Svedberg constants, and the partial specific volumes. The values estimated for monosialo-gangliosides with M_r of 280,000 to 340,000 were close to those determined by sedimentation analysis by Gammack (4), Yohe, Roark, and Rosenberg (3) and Yedgar, Barenholz, and Cooper (26). In contrast, Corti et al. (19) with light scattering measurements and Tomasi et al. (27) using gel filtration reported much higher relative molecular masses for ganglioside $G_{tet}1$-

micelles, i.e., 532,000 and 450,000. On the other hand, the relative molecular masses for ganglioside $G_{tet}2a$ (M_r 181,000). $G_{tet}2b$ (M_r 160,000), and $G_{tet}3b$ (M_r 113,000), respectively, are much lower than those given earlier by Yohe et al. ((3), M_r 300,000 $G_{tet}2$ and M_r 250,000 for $G_{tet}3$) and Corti et al. ((19), M_r 417,000 for $G_{tet}2a$).

The present study confirms the earlier notion that GSL's, including the easily water-soluble gangliosides, show strong self-aggregation properties in aqueous systems that are not only governed by their lipophilic ceramide portion but also strongly influenced in a subtle way by hydrophilic interactions. This behavior of GSL may well be of great importance for their properties in biological membranes.

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REFERENCES

1. Wiegandt, H. 1982. The gangliosides. *Adv. Neurochem.* **4:** 149–223.
2. Yohe, H. C., and A. Rosenberg. 1972. Interaction of triiodide anion with gangliosides in aqueous media. *Chem. Phys. Lipids.* **9:** 279–294.
3. Yohe, H. C., D. E. Roark, and A. Rosenberg. 1976. C_{20}-sphingosine as a determining factor in aggregation of gangliosides. *J. Biol. Chem.* **251:** 7083–7087.
4. Gammack, D. B. 1963. Physico-chemical properties of ox-brain gangliosides. *Biochem. J.* **88:** 373–383.
5. Formisano, S., G. Lee, S. M. Aloj, and H. H. Edelhoch. 1979. Critical micellar concentration of gangliosides. *Biochemistry* **18:** 1119–1124.
6. Mraz, M., G. Schwarzmann, J. Sattler, B. Seemann, T. Momoi, and H. Wiegandt. 1980. Aggregate formation of gangliosides at low concentrations in aqueous media. *Hoppe-Seyler's Z. Physiol. Chem.* **361:** 177–185.
7. Ledeen, R. W., R. R. Yu, and K. F. Eng. 1973. Gangliosides of human myelin, sialogalactosylceramide (G_7) as a major component. *Neurochemistry* **21:** 829–839.
8. Momoi, T., and H. Wiegandt. 1980. Preparation and microdetection of oligosaccharides of glycosphingolipids by high performance cellulose thin-layer chromatography–autoradiofluorography. *Hoppe-Seyler's Z. Physiol. Chem.* **361:** 1201–1210.
9. Watanabe, K., and Y. Arao. 1981. A new solvent system for the separation of neutral glycosphingolipids. *J. Lipid Res.* **22:** 1020–1024.
10. Ohsawa, T., K. Nakane-Hikichi, and Y. Nagai. 1984. Alteration of ceramide monohexoside in human diploid fetal lung fibroblasts during cell aging. *Biochim. Biophys. Acta.* **792:** 79–83.
11. Miettinen, T., and J-J. Takki-Luukainen. 1959. Use of butyl acetate in determination of sialic acid. *Acta Chem. Scand.* **13:** 856–858.
12. Svedberg, T., and K. O. Peterson. 1940. Die Ultrazentrifuge. Th. Steinkopf, Dresden.
13. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236:** 1372–1379.
14. Landolt-Börnstein. 1923. Physikalisch-Chemische Tabellen. J. Springer, Berlin.
15. Bates, F. Y. 1944. Polarimetry, Saccharimetry and the Sugars. U.S. Government Printing Office, Washington.
16. Tanford, C., J. Nozaki, A. Reynolds, and S. Mukino. 1974. Molecular characterization of proteins in detergent solutions. *Biochemistry* **13:** 2369–2376.
17. Iwamori, M., and Y. Nagai. 1978. A new chromatographic approach to the resolution of individual gangliosides. Ganglioside mapping. *Biochim. Biophys. Acta.* **528:** 257–267.
18. Howard, K. E., and R. M. Burton. 1964. Studies on the ganglioside micelle. *Biochim. Biophys. Acta.* **84:** 435–448.
19. Corti, M., V. DeGorgio, R. Ghidoni, S. Sonnino, and G. Tettamanti. 1980. Laser-light scattering investigation of the micellar properties of gangliosides. *Chem. Phys. Lipids.* **26:** 225–238.
20. Lipovac, V., G. Bigalli, and A. Rosenberg. 1971. Enzymatic action of sialidase of *Vibrio cholerae* on brain gangliosides above and below the critical micelle concentration. *J. Biol. Chem.* **246:** 7642–7648.
21. Rauvala, H. 1976. Action of *Clostridium perfringens* neuraminidase on gangliosides G_{M1} and G_{M2} above and below the critical micelle concentration of substrate. *FEBS Lett.* **65:** 229–233.
22. Rauvala, H. 1979. Monomer-micelle transition of the ganglioside G_{M1} and the hydrolysis by *Clostridium perfringens* neuraminidase. *Eur. J. Biochem.* **97:** 555–564.
23. Mraz, M., G. Schwarzmann, J. Sattler, B. Seemann, T. Momoi, and H. Wiegandt. 1979. Aggregate formation of gangliosides in water. Glycoconjugates, Proc. Vth Intl. Symposium Kiel, Thieme Verlag, Stuttgart. 112–113.
24. Heuser, E., K. Lipp, and H. Wiegandt. 1974. Detection of sialic acid containing compounds and the behaviour of gangliosides in polyacrylamide disc electrophoresis. *Anal. Biochem.* **60:** 382–388.
25. Tettamanti, G., A. Preti, B. Cestaro, M. Massernini, S. Sonnino, and R. Ghidoni. 1980. Gangliosides and associated enzymes at the nerve-ending membranes. ACS, Symposium Series. **128:** 321–343.
26. Yedgar, S., Y. Barenholz, and V. G. Cooper. 1974. Molecular weight, shape and structure of mixed micelles of Triton X-100 and sphingomyelin. *Biochim. Biophys. Acta.* **363:** 98–104.
27. Tomasi, M., L. G. Roda, C. Ausiello, G. D'Angelo, B. Venerando, R. Ghidoni, S. Sonnino, and G. Tettamanti. 1980. Interaction of G_{M1} ganglioside with bovine serum albumin, formation and isolation of multiple complexes. *Eur. J. Biochem.* **111:** 315–324.