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Critical Micelle Concentrations of Gangliosides†

Silvestro Formisano,‡ Michael L. Johnson, George Lee, Salvatore M. Aloj,‡ and Harold Edelhoch*

ABSTRACT: The micellar properties of mixed, bovine gangliosides and purified galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide were studied by gel filtration, equilibrium dialysis, and band and boundary centrifugation in sucrose gradients. The dissociation of micelles is very slow (days) in water and required us to approach equilibrium by association of monomers rather than by the dissociation of micelles. The gangliosides were therefore first converted into very low molecular weight aggregates (1–3 molecules) by dissolving them in Me_2SO. Galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide was then diluted into aqueous sucrose gradients and sedimented by the boundary centrifugation technique. This gave a sedimenting micelle and a nonsedimenting monomer concentration of (1–2) × 10^{-10} M (or less) which corresponds to the critical micelle concentration value. The mixed gangliosides revealed two micellar sizes (i.e., 10 and 4.5 S), the slower sedimenting species being formed from the larger one with time (days). The critical micelle concentration of the mixed gangliosides was found to be approximately 10^{-8} M by gel filtration, equilibrium dialysis, and band centrifugation.

Gangliosides were originally described as acylsphingosyl oligosaccharides containing sialic acid (Klenk, 1942). It was later shown that higher order gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1}, etc.)[1] have a common basic structure, i.e., *N*-acylsphingosineglucosegalactose-*N*-acetylgalactosaminegalactose, with one or more molecules of *N*-acetylneuraminic acid (Svennerholm, 1962). G_{M2} and G_{M3} are biosynthetic precursors with deletions at the terminal galactose and galactosaminegalactose residues, respectively.

Gangliosides have been implicated in the binding or function of many biologically important molecules, i.e., cholera toxin (Sattler et al., 1977), tetanus toxin (Helting et al., 1977), botulinum toxin (Haberman & Heller, 1975), thyrotropin (Mullin et al., 1976), human chorionic gonadotropin (Lee et al., 1976), luteinizing hormone (Lee et al., 1977), serotonin (Ochoa & Bangham, 1976), interferon (Vengris et al., 1976), bilirubin (Kahan et al., 1968), and wheat germ agglutinin (Redwood & Polefka, 1976).

Table I: Literature Values for the cmc of Gangliosides

cmc (M)	ganglioside	technique	reference
1×10^{-4}	mixed	surface tension	Gammack (1963)
1×10^{-5}	mixed	conductance	Howard & Burton (1964)
1×10^{-4}	G_{M1}	neuraminidase	Rauvala (1976)
7.5×10^{-5}	G_{M2}	triiodide method	Yohe & Rosenberg (1972)
8.5×10^{-5}	G_{M1}	triiodide method	Yohe & Rosenberg (1972)
9.5×10^{-5}	G_{D1a}	triiodide method	Yohe & Rosenberg (1972)
1×10^{-4}	G_{T1}	triiodide method	Yohe & Rosenberg (1972)

The interpretation of studies involving gangliosides depends on a knowledge of their state of aggregation since the observed biological effects occur, in many cases, in the concentration range of the reported cmc values, i.e., 10^{-4}–10^{-5} M (see Table I). Unfortunately, none of the cmc values was obtained by use of a technique which directly measured either the size or the molecular weight of the gangliosides. It has also been observed that at concentrations below these values purified preparations of G_{M1} and G_{M2} were unable to cross a dialysis membrane (Kanfer & Spielvogel, 1973; Sattler et al., 1977). In an attempt to resolve this apparent contradiction, we undertook a study of the molecular behavior of typical gangliosides.

Materials and Methods

Mixed, bovine brain gangliosides were purchased from ICN Pharmaceuticals. The composition was measured by thin-layer chromatography (TLC) on silica gel plates and is shown in Figures 1 and 2. Purified G_{M1} was either purchased from Supelco or obtained by a purification of the mixed gangliosides

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[1] Abbreviations used: cmc, critical micelle concentration; Me_2SO, dimethyl sulfoxide; ganglioside nomenclature is according to Svennerholm (1964)—G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{D1b}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{T1}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylcerimide; G_{M3}, *N*-acetylneuraminylgalactosylglucosylcerimide.

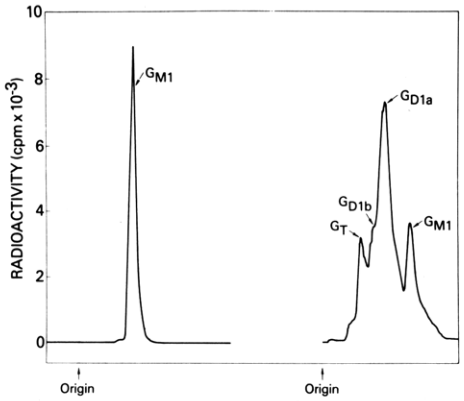

FIGURE 1: Radioscans of the labeled ganglioside preparations after thin-layer chromatography as in Figure 2. The radioscan of G_{M1} is on the left and the radioscan of the mixed, brain ganglioside preparation is on the right.

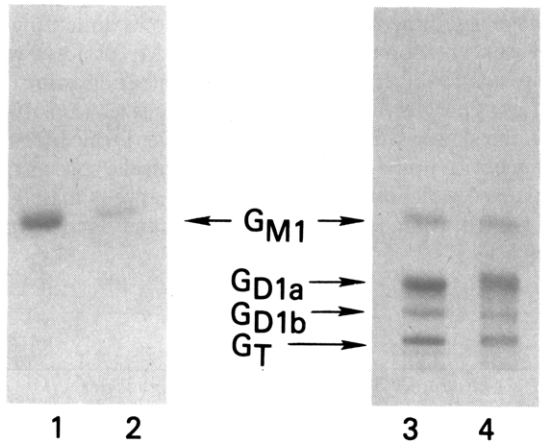

FIGURE 2: Thin-layer chromatography, on silica gel plates in chloroform–methanol–0.25% $CaCl_2$ (60:35:8 v/v/v), after resorcinol staining: (1) G_{M1} before tritiation; (2) G_{M1} after tritiation; (3) mixed, bovine brain gangliosides before tritiation; and (4) mixed, bovine brain gangliosides after tritiation.

when we followed the procedure of Fishman et al. (1974). The purity of the G_{M1} was evaluated by TLC and shown to migrate as a single band (Figures 1 and 2). Concentrations of gangliosides were determined by their sialic acid content by use of a micromodification of the resorcinol method of Svennerholm (1957).

Gelatin was purchased from Nutritional Biochemicals. Tritiated sodium borohydride was purchased from Amersham. All other chemicals were reagent grade and glass-distilled water was used for all solutions. All solutions contained 10^{-4} M sodium azide.

Tritiated gangliosides were made by mild periodate oxidation and subsequent reduction with ^{3}H-labeled sodium borohydride when we followed the procedure of Blumenfeld et al. (1972). Figures 1 and 2 demonstrate that this procedure does not affect the composition as measured by TLC. Radioscans of TLC plates were performed by use of a Berthold TLC scanner (time constant 50 s; scanning speed 300 mm/h).

Equilibrium dialysis experiments were performed in plexiglass cells with equal volumes (1 mL) on the two sides of the membrane. The cells were constantly agitated during the course of the experiments. At various times equal volumes were removed from both sides of the membrane for radioactivity measurements.

"Low-speed" sedimentation equilibrium experiments at 52000 rpm and 24.8 °C were performed in a Beckman Model E ultracentrifuge with an Arden Instruments temperature controller. Z-average molecular weights were determined by use of Schlieren optics and the relationship

$$M_Z = \frac{RT}{(1-\bar{v}\rho)\omega^2} \frac{\mathrm{d}\ln\left(\frac{1}{r}\left(\frac{\mathrm{d}c}{\mathrm{d}r}\right)\right)}{\mathrm{d}(r^2/2)}$$

The solvent was Me_2SO containing 0.1 M KI. The density of this solvent, measured at 24.8 °C by pycnometry, was 1.108 g/mL.

Density-gradient sedimentation was performed in two ways: (1) when we layered a small amount of sample at the top of a preformed sucrose gradient, i.e., band centrifugation, and (2) when we diluted the gangliosides into the two sucrose solutions before the gradient was formed, i.e., boundary centrifugation. In boundary centrifugation the gangliosides are initially evenly distributed throughout the gradient.

Sedimentation rates were calculated from the sucrose gradient experiments by the method of Martin & Ames (1961). The partial specific volume of G_{M1} was calculated by the method of Cohn & Edsall (1943) as applied to phospholipids by Tanford & Reynolds (1976) and to sugars by Gibbons (1972). The results are presented in Table II along with the values for other naturally occurring gangliosides. For mixed, bovine brain gangliosides we used the measured value of $\bar{v}$, i.e., 0.78 mL/g (Gammack, 1963).

Fluorescence measurements were made on a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Fluorescence was used as a measure of the position of various proteins in the sucrose gradients by exciting at 280 nm and monitoring the emission at 340 nm.

Table II: Partial Specific Volumes of the Individual Gangliosides[a]

ganglioside	$\bar{v}$ (mL/g)	ganglioside	$\bar{v}$ (mL/g)
G_{M1}	0.781	G_{D1b}	0.752
G_{M2}	0.798	G_{D2}	0.763
G_{M3}	0.816	G_{D3}	0.772
G_{D1a}	0.752	G_{T1}	0.731

[a] Calculated by the method of Cohn & Edsall (1943) as applied to phospholipids by Tanford & Reynolds (1976) and to sugars by Gibbons (1972).

Results

(*A*) *Gel Filtration.* The gel filtration elution profile of a monomer–micelle equilibrium will show an asymmetrical boundary. The shape of the trailing boundary is determined by the rate of resolution compared to the rate of equilibration between monomer and micelle molecules (Herries et al., 1964). If the rate of dissociation of micelles is rapid compared with rate of filtration, the trailing boundary will appear as a "plateau" at a concentration corresponding to the cmc. However, if the rate of dissociation is slow, the trailing boundary will be resolved into a separate peak. The cmc can be evaluated from the height of this trailing boundary, if we assume that the boundary is sufficiently broad that a "plateau" exists. This is analogous to the procedure used by Josephs & Harrington (1967) to measure the monomer to approximately 80-mer association of myosin.

Gel filtration patterns of labeled, mixed gangliosides on Bio-Gel P-20 are presented in Figure 3. The molecular weight of ganglioside micelles has been reported to be between 250000 and 446000 when measured by sedimentation equilibrium (Yohe et al., 1976) or by sedimentation, diffusion, and viscosity

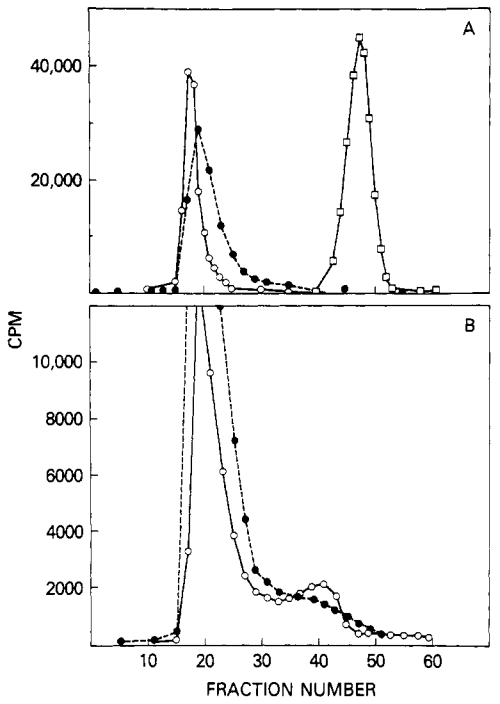

FIGURE 3: Gel filtration elution profile of labeled, mixed gangliosides (●) on a Bio-Gel P-20 column (60 × 0.9 cm). Fraction volumes were approximately 0.75 mL, the flow rate was 0.15 mL/min, and the temperature was 24 °C. (A) Load was 4.5 mL of 3×10^{-7} M gangliosides dissolved in 0.02 M Tris titrated to pH 7.4 with acetic acid, 10^{-4} M sodium azide. Also shown are 19S thyroglobulin (○) (excluded volume) and *N*-acetyl-L-tryptophanamide (□) (included volume). (B) Gel filtration elution profile of labeled, mixed gangliosides plotted on an expanded scale. The open circles correspond to a load of 2.5 mL of 3×10^{-7} M gangliosides which had been diluted 12 days before the experiment and stored at room temperature.

(Gammack, 1963); consequently, the micellar form of the gangliosides is expected to be at the excluded volume of the column. The elution profiles of 19S thyroglobulin and *N*-acetyltryptophanamide are given in Figure 3A and serve as a measure of the excluded and included volumes of the column. Any gangliosides which are not excluded from the column must be either monomers or small aggregates since the exclusion limit of Bio-Gel P-20 is approximately 20 000 daltons. The ganglioside peak in Figure 3A is only slightly displaced from the marker which defines the excluded volume, indicating that it is excluded from the column and is thus micellar. The ganglioside data of Figure 3A are presented in Figure 3B on an expanded scale in order to show the trailing boundary in more detail. This experiment was repeated 12 days after the sample was diluted to 3×10^{-7} M. To improve the resolution on the column, a smaller loading volume was used. In this case, a peak was observed in the same region as the shoulder in the previous experiment (Figure 3B). Both experiments gave essentially the same result, i.e., a trailing peak of radioactivity corresponding to a plateau concentration of about 8.5×10^{-9} M. If any low molecular weight radioactive contaminant is present, it would also be expected to be retarded on the column and could occur in this trailing boundary. In such a case the height of this boundary would be an overestimate of the cmc value. In the experiment with the smaller initial loading volume, a distinct peak was found for the trailing boundary, indicating that the rate of dissociation of micelles was slow compared with the duration of the experiment. It is possible that the dissociation is still incomplete after 12 days, in which case the cmc would be larger than 8.5×10^{-9} M.

(*B*) *Equilibrium Dialysis.* The results from the gel filtration experiments indicated that the rate of dissociation of ganglioside micelles is quite slow. Equilibrium dialysis experiments were performed to confirm these results.

Equilibrium dialysis experiments of a monomer–micelle equilibrium could show several kinds of results depending on the rate of dissociation of micelles. If the rate were very slow compared to the rate of dialysis, only the dilution of the monomer into the solvent side of the membrane would be observed. If the micelle dissociation rate were fast, a rapid rise in concentration to the cmc would occur. Intermediate rates would predict a rapid rise to the concentration predicted by the dilution, as in the case of a slow dissociation, and then a slow rise to the cmc. The rate of this slow rise would be a direct measure of the dissociation rate of the micelles.

An equilibrium dialysis experiment of mixed, labeled gangliosides was performed in 0.2 M Tris, pH 7.4, with 10^{-4} M sodium azide (data not shown). The samples were diluted 16 days prior to the experiment in order to minimize the possibility of slow time effects. At a ganglioside concentration of 1.4×10^{-8} M, approximately 26% of the radioactivity crossed the membrane. At 7.5×10^{-8} M, 5.6% of the radioactivity crossed the membrane. In both of these cases the stated level was reached within 0.5 h, and no subsequent increase was observed for a period of 48 h. Furthermore, the concentration which crossed the membrane was essentially the same for both of these concentrations. At initial concentrations of 8×10^{-7} and 8×10^{-6} M approximately 1% of the total radioactivity rapidly crossed the dialysis membrane. This small percentage was interpreted to be due to a contaminant not involved in the micelle equilibrium. When we allowed for the 1% contaminant, the radioactivity which crossed the membrane corresponds to 3.5×10^{-9} M gangliosides for initial loading concentrations of 1.4×10^{-8} and 7.5×10^{-8} M. At these two loading concentrations a significant fraction of the radioactivity crossed the membrane, and the final concentration on the outside of the membrane was constant, in accord with the result expected for a monomer–micelle equilibrium.

There was no significant increase in radioactivity crossing the membrane between 0.5 and 50 h. This suggests the following possibilities: if the dissociation is complete in 16 days, then the cmc is twice 3.5×10^{-9} (7×10^{-9} M), or if the dissociation rate is slower than 16 days, then 7×10^{-9} M is a lower limit for the cmc. Consequently, the cmc of mixed, labeled gangliosides is greater than or equal to 7×10^{-9} M.

(*C*) *Sedimentation Studies.* In both the gel filtration and dialysis experiments the possibility exists that the dissociation rate is very slow, and consequently a time period of 12–16 days is not sufficient to establish equilibrium. However, if micelles could be formed by associating ganglioside monomers, equilibrium would be assured. This was accomplished when we dissolved the gangliosides in Me_2SO and then diluted the mixture into the desired aqueous buffer (final Me_2SO concentration being <1%).

In order to be certain that the gangliosides were monomeric or highly dissociated in Me_2SO, we have determined the Z-average molecular weight of unlabeled, mixed gangliosides by sedimentation equilibrium at 52 000 rpm. The solution included 0.1 M KI to minimize the effects of the Donnan equilibrium and to establish a stabilizing density gradient (Rees & Singer, 1956). The initial loading concentration was approximately 3 mg/mL. A plot of $\ln[(1/r)(dc/dr)]$ vs. $r^2/2$ was linear and gave a Z-average molecular weight of 3500 ± 500 by use of the literature value of $\bar{v}$, 0.78 mL/g for mixed gangliosides (Gammack, 1963). When we used the extreme values of the calculated $\bar{v}$, as presented in Table II, the Z-average molecular weight would be 4900 ± 700 for G_{M3} and

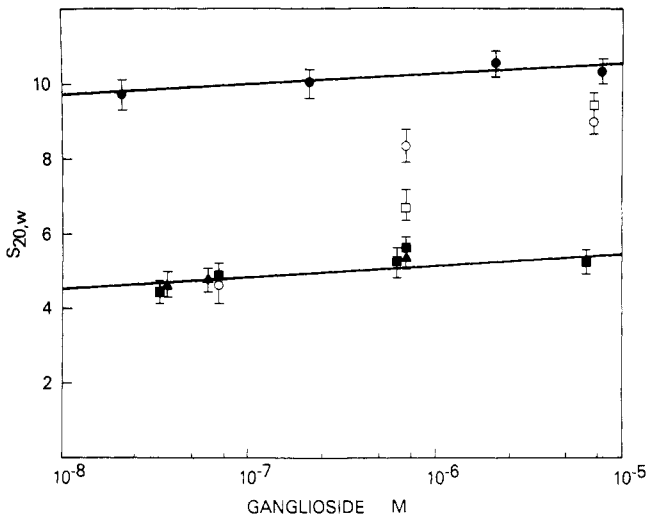

FIGURE 4: Band sedimentation rates of labeled, mixed gangliosides (mol/L) as a function of concentration. Symbols correspond to the following: (▲ and ■) samples were dissolved in Me_2SO, diluted into aqueous buffer, and centrifuged immediately; (●) samples were dissolved in aqueous buffer, diluted, and centrifuged within 3 days; (○) 15 days later; (□) 20 days later. Sedimentation rates were determined by the method of Martin & Ames (1961), by use of either 5–15% or 5–25% sucrose gradients, at 22 °C, and a partial specific volume of 0.78 mL/g (Gammack, 1963). Buffer was 0.02 M Tris titrated to pH 7.4 with acetic acid, 10^{-4} M sodium azide.

2500 ± 350 for G_{T1}. The gangliosides are therefore only very slightly associated in Me_2SO since the Z-average molecular weight strongly weights the largest molecules. Consequently, if micelles are observed after dilution from Me_2SO into water, they must have been formed by reassociation of monomers.

The band sucrose density centrifugation procedure (i.e., when we layered the sample on top of the gradient) would predict for a monomer–micelle equilibrium the same pattern as for gel filtration, i.e., a slowly sedimenting shoulder of monomer, a rapidly sedimenting peak of micelle and a plateau between the two. This plateau would be at the monomer concentration, i.e., the cmc, for a rapid monomer–micelle equilibrium, significantly less than the cmc for a very slow rate of dissociation, and at an intermediate value if the rate of dissociation is comparable to the time scale of the experiment.

Sedimentation coefficients of mixed, labeled gangliosides at concentrations between 2×10^{-8} and 9×10^{-6} M were calculated from the boundaries obtained by band centrifugation (see Figure 4). The gangliosides were dissolved in aqueous buffer, diluted, and then centrifuged immediately on a sucrose gradient. With this procedure $s_{20,w}$ values of approximately 10 were found at concentrations as low as 2×10^{-8} M (Figure 4). This sedimentation rate is in agreement with the literature value of 10.3 ± 0.2 S (Gammack, 1963). When the gangliosides were first dissolved in Me_2SO and then diluted into buffer (Me_2SO, <1%) and immediately centrifuged on a sucrose gradient, $s_{20,w}$ values of approximately 4.5 were observed (Figure 4). When they were directly dissolved in aqueous buffer and then stored at 22 °C for 15 or 20 days (in azide) prior to gradient centrifugation, the sedimentation rates were less than 10 and approached 4.5 S (Figure 4). Consequently, it appears that after 15–20 days in dilute aqueous solution the 10S micelle is transformed to a slower sedimenting micelle.

When the band centrifugation procedure was performed with labeled, mixed gangliosides (data not shown), a very low level of radioactivity was found in the region following the micelle peak, indicating a slow dissociation or a very low cmc.

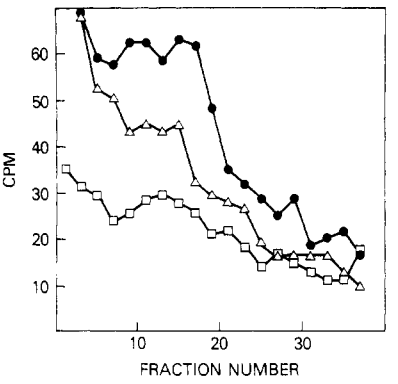

FIGURE 5: Boundary sucrose density gradient sedimentation of G_{M1}. The 5–25% gradient was formed after the gangliosides were dissolved in Me_2SO and then diluted more than 100-fold into the individual sucrose solutions. Solutions were centrifuged for 26 h at 35 000 rpm and 22 °C. Buffer was 0.02 M Tris titrated to pH 7.4 with acetic acid, 0.1 M NaCl, 10^{-4} M sodium azide. Initial loading concentrations were 4×10^{-10} (●), 3×10^{-10} (△), and 2×10^{-10} M (□), $s_{20,w}$ is 6.5 ± 0.5 S.

Since the ganglioside monomers would be found in the last tube of the sucrose gradient fractions due to their small sedimentation rate (Gammack, 1963), it is difficult to obtain their concentration, and thus the cmc, precisely. It is, however, obvious that, since a significant amount of mixed gangliosides reassociated to approximately 4.5 S upon transfer from Me_2SO to aqueous buffer at 2×10^{-8} M, the cmc must be lower than 2×10^{-8} M, the lowest concentration used in these experiments.

A second procedure was applied in order to obtain a value of the free monomer concentration since it is difficult to assess the nonsedimenting monomer concentration by band centrifugation. In this procedure the purified preparation of G_{M1} was diluted from Me_2SO into the two sucrose solutions before the gradient was prepared. This gives a distribution of gangliosides which is constant throughout the tube; thus the sedimentation pattern resembles that obtained by boundary sedimentation in the analytical ultracentrifuge. In these experiments the nonsedimenting radioactivity would correspond to the concentration of free monomer, i.e., the cmc. It should be noted that, if a nonsedimenting contaminant is present, it would appear as monomer.

A sucrose density gradient boundary sedimentation experiment of labeled, purified G_{M1} at concentrations of 4×10^{-10}, 3×10^{-10}, and 2×10^{-10} M is presented in Figure 5. The experiment showed the expected behavior of a monomer–micelle equilibrium and the amount of monomer concentration or cmc of approximately 10^{-10} M.

In numerous experiments the recovery of radioactivity was variable, especially at the lower ganglioside concentrations. Consequently, an attempt was made to find a substance which would inhibit the binding of gangliosides to the walls of the vessels with which the dilute solutions came into contact and also which did not interact with the gangliosides. We observed that bovine serum albumin and G_{M1} sedimented as a single boundary at a concentration of 2×10^{-8} M, revealing a strong interaction (Figure 6). Bovine serum albumin could therefore not be used to reduce adsorption to the walls. Similar interactions were found with two other proteins, ovalbumin and fumarase. Gelatin, however, did not show any interaction and we consequently used a buffer containing 0.20% gelatin.

Figure 7 presents a sucrose density gradient boundary sedimentation profile of a different preparation of labeled G_{M1} (performed as in Figure 5) in the presence of 0.20% gelatin. The variability observed in the absence of gelatin was no longer

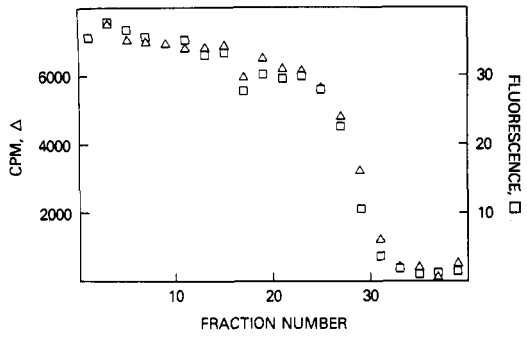

FIGURE 6: Boundary sucrose density gradient sedimentation of a mixture of G_{M1} and bovine serum albumin. Solutions were centrifuged for 16 h at 35000 rpm and 22 °C. (□) Bovine serum albumin at an initial concentration of 0.02% and monitored by 340-nm fluorescence; (△) labeled G_{M1} at an initial concentration of 2×10^{-8} M. Other conditions are as in Figure 5.

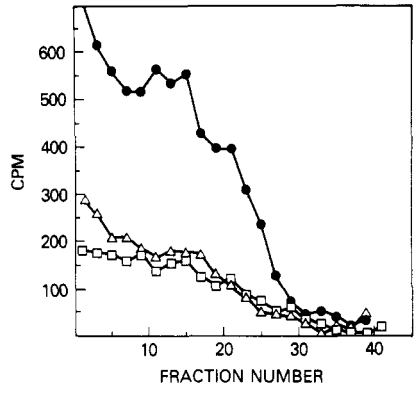

FIGURE 7: Boundary sucrose density gradient sedimentation of G_{M1} in 0.20% gelatin. Solutions were centrifuged for 16.5 h. Initial loading concentrations were approximately (●) 3×10^{-9}, (△) 1×10^{-9}, and (□) 8×10^{-10} M. Other conditions are as in Figure 5. $s_{20,w}$ is 8.8 ± 0.9 S.

observed, indicating that gelatin had eliminated surface effects. The cmc of this preparation, as measured by the nonsedimenting radioactivity, is less than 2×10^{-10} M.

Discussion

In a thorough study of the kinetics of micelle association/dissociation rates, it has recently been shown by Aniansson et al. (1976) that the rate of association of monomers to micelles in the sodium alkyl sulfate series $Na(CH_2)_nSO_4$ is almost independent of chain size and is essentially diffusion controlled between $n = 6$ and 12. With longer chain length molecules in this series ($n = 14$ and 16), the association rates decrease. The dissociation rate therefore decreases regularly with chain length between $n = 6$ and 12 and follows the changes in the cmc values. If the dissociation rate of the gangliosides is similarly related to their cmc values, a very slow rate would be expected considering their extremely low cmc values. A dissociation rate of several hours has been observed for lysolecithin (Schneider & Edelhoch, 1972). The dissociation rate of gangliosides would be expected to be much lower since the ganglioside cmc values are several orders of magnitude lower than that reported for lysolecithin, i.e., 7×10^{-6} M (Haberland & Reynolds, 1975). The rate of dissociation of micelles of mixed gangliosides can be approximated from some of the experiments that have been performed. For instance, a minimum rate, i.e., the time to pass through the column, can be deduced from the gel filtration experiments since the experiment performed with the smaller loading volume was resolved into two peaks. It is evident from the equilibrium dialysis experiments that the rate of dissociation is greater than 50 h since no increase in radioactivity, i.e., concentration of mixed gangliosides, was observed outside the dialysis membrane between 0.5 and 50 h.

Since the sedimentation coefficients of the mixed ganglioside micelles depend on whether solutions were prepared from water or Me_2SO, it appears that two distinct micellar sizes, i.e., 10 and 4.5 S, may exist for these compounds. It was also shown that the size of the 10S micelle decreased with time and approached that of the 4.5S micelle after 15–20 days. It should be kept in mind that the larger species, i.e., the 10S micelle, may be some artifact of the method of its final precipitation when the sample is prepared commercially. A preliminary report by Haberland & Reynolds (1975) indicates two types of micelles may also exist for lysolecithin.

A frictional ratio of 1.4 can be calculated from the average micellar molecular weight determined by Yohe et al. (1976), i.e., 350000, and the observed sedimentation rate of 10 S. This value is consistent with the diffusion and viscosity measurements of Gammack (1963). When we used the same frictional ratio, a 4.5S micelle would correspond to approximately 100000 molecular weight.

The association of monomers (or very small aggregates) to micelles should be very fast. The 4.5S particles observed when Me_2SO solutions of gangliosides are diluted into water should therefore represent the smallest micelle observable in water. It is evident that this species is the only equilibrium micelle since the 10S particle observed when we dissolved the mixed gangliosides directly into water dissociates to the 4.5S particle in 15 days at concentrations below 10^{-7} M. The solutions used for equilibrium dialysis had been diluted to their final concentrations 16 days before dialysis and should be near equilibrium. Consequently, these experiments indicate that the cmc is near the experimentally determined values of 8.5×10^{-9} M (gel filtration) and 7×10^{-9} M (equilibrium dialysis). The sedimentation velocity studies on mixed, labeled gangliosides diluted from Me_2SO_4 indicate that the cmc is less than 2×10^{-8} M. The agreement between the upper (2×10^{-8} M) and lower [$(7–8.5) \times 10^{-9}$ M] limits, as determined by different techniques, for the cmc of mixed gangliosides indicates that, if the gangliosides were diluted 15–20 days prior to observation, the monomer–micelle dissociation would be near equilibrium. It should be noted that these cmc values are several orders of magnitude lower than those previously reported (see Table I).

A few experiments were performed with the purified ganglioside G_{M1} by boundary centrifugation. In this case we started with G_{M1} dissolved in Me_2SO so that the sedimenting boundary represents reassociated micelles and there should be no problem with equilibrium. In two series of experiments (Figures 5 and 7) the nonsedimenting radioactivity corresponds to $(1–2) \times 10^{-10}$ M. If the possibility of a small amount of a labeled contaminant is included, this value would be an upper estimate of the cmc for G_{M1}. A value similar to this was found for dipalmitoylphosphotidylcholine (Smith & Tanford, 1972), a molecule having a backbone structure, i.e., two long hydrocarbon chains, similar to that of G_{M1}.

This value for the cmc of G_{M1} is more than five orders of magnitude less than the literature value (see Table I). It is difficult to explain the discrepency between our results and the previously reported values. One possible explanation, however, is that none of the previous measurements was performed by a technique which measures directly the size of the micelle. While these techniques are capable of demonstrating a transition to a different molecular species, the exact

nature of this transition is ambiguous. In the case of the surface tension and conductance measurements, listed in Table I, the transition is to a lower molecular weight species, but this species was not demonstrated to be monomeric. The other experiments listed in Table I have no direct interpretation in terms of molecular size.

Almost all published studies on the interactions of the gangliosides, either pure or mixed, with hormones, toxins, etc., therefore pertain to micelles rather than to monomer since the cmc values are extremely small and the rate of dissociation of the micelles is very slow.

In an attempt to decrease the amount of nonspecific binding, we tested the effect of bovine serum albumin, ovalbumin, and fumarase in the aqueous buffer solution. In all of these cases we observed an apparent interaction between the ganglioside and the protein (see Figure 6). This confirms the report of Hayashi & Katagiri (1974) of an albumin–ganglioside complex. Bovine serum albumin has also been found to affect the inhibition of thyrotropin binding by G_{D1a} and G_{M1} (Aloj et al., unpublished experiments). Consequently, care should be taken to include the effects of this interaction in any studies of gangliosides when an interacting protein is present. Gelatin appears to be much better than bovine serum albumin, ovalbumin, or fumerase as an indifferent protein to coat the walls of the vessels used to study ganglioside behavior.

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