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PHASE BEHAVIOR AND STRUCTURAL CHARACTERISTICS OF HYDRATED BOVINE BRAIN GANGLIOSIDES

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Summary

Hydrated bovine brain gangliosides have been studied by differential scanning calorimetry, X-ray diffraction, and polarized light microscopy. Over the hydration range 18—50 wt.% H_2O, mixed brain gangliosides exhibit a hexagonal mesophase structure, in which the ganglioside molecules form hexagonally packed rod-like structures. The apolar lipid chains radiate from the center of the rods, with the sugar groups on the cylinder surface in contact with water. At higher water contents, an isotropic micellar solution is formed.

Over the hydration range 20—30 wt.% H_2O, two small thermal transitions with peak maxima at 30°C and 46°C are observed by differential scanning calorimetry. These transitions broaden and move apart in temperature as the hydration is increased to 50 wt.% H_2O. X-ray diffraction data indicate that this double transition is associated with a hydrocarbon chain rearrangement from a disordered state to another, possibly more disordered, state. Thus, the gangliosides, although membrane lipid components, have physical characteristics which are very different from those of the membrane phospholipids.

Introduction

The gangliosides are sialic acid-containing glycosphingolipids [1] which have been shown to be involved in a variety of cell surface phenomena. These complex molecules can serve as receptors for cholera toxin [2], tetanus toxin [3], and Sendai virus [4], and have been shown to change in composition in cultured cells after cell-cell contact and transformation [5]. The absence of

Abbreviations: G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl-ceramide: G_{D1b}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)$_2$-galactosylglucosylceramide; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl-ceramide.

enzymes involved in the catabolism of the gangliosides and other glycosphingolipids results in a series of hereditary sphingolipidoses, or glycolipid storage diseases, in which large amounts of individual glycosphingolipids accumulate, resulting in severe disruption of the central nervous system and other tissues. (For a review see ref. 6.)

The physical properties of the gangliosides have received little attention, probably because of the difficulty in obtaining large amounts of these compounds. Gammack [7] observed that mixed brain gangliosides in dilute aqueous solution form prolate ellipsoidal micelles (axial ratio $\approx$ 2) of molecular weight 2.5—$4.0 \cdot 10^5$, as determined from sedimentation, diffusion, and viscosity measurements. A critical micelle concentration of 0.15% was determined by surface tension [7]. Hilland Lester [8] studied phosphatidylcholine/ganglioside mixtures by ultracentrifugation and observed that at low ganglioside/phosphatidylcholine ratios, the ganglioside sedimented with the phosphatidylcholine, indicating that the gangliosides were incorporated into the phospholipid bilayers. At high ganglioside/phosphatidylcholine ratios the phospholipid and ganglioside were found in the supernatant, presumably as a mixed micelle. At intermediate ratios an undefined phase or mixture of phases was present. The gangliosides are atypical amphipathic membrane lipids, comprised of polar head group and apolar acyl chain regions of approximately equal volume. Inspection of a space-

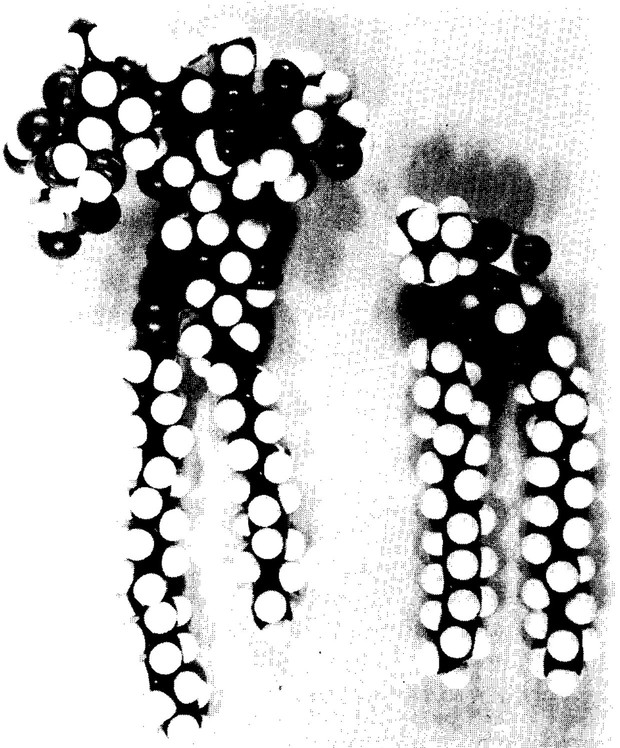


Fig. 1. Corey-Pauling-Koltun models of ganglioside G_{D1a} (left) and phosphatidylcholine (right).

filling molecular model of a ganglioside emphasizes the potentially unusual physical characteristics of this molecule (Fig. 1). A knowledge of the interactions between these complex glycosphingolipids and other membrane lipids is essential for an understanding of the role of gangliosides both in normal membrane phenomena and in pathological accumulations. As a prerequisite to this type of understanding, studies on the interactions of gangliosides with water are reported in this paper.

Materials and Methods

Bovine brain gangliosides (Type III) were purchased from Sigma, St. Louis, Mo. (lot Nos. 44C-8050, 74C-8280) and were judged pure by thin layer chromatography, except for small amount of sulfatide ($\approx$ 1%). Comparison with homogenous ganglioside standards (generously provided by Dr. R.H. McCluer, Shriver Institute, Waltham, Mass.) showed the mixed bovine brain gangliosides to consist primarily of G_{D1a}, G_{D1b}, G_{M1}, and a small amount of G_{M2}. All solvents were spectroanalyzed grade. Water was doubly distilled.

Samples were prepared by weighing out mixed gangliosides in powder form into a glass tube. Sufficient doubly distilled water was added to dissolve the gangliosides to an optically clear micellar solution. The tube was then attached to a rotary evaporator and the water was slowly removed at 22 ± 2°C. As the water evaporated, the sample first became viscous, and later a visible change to a gel took place. The evaporation was continued for various periods of time to vary the water content. Aliquots were removed for differential scanning calorimetry, X-ray diffraction, polarized light microscopy, and water content determination by gravimetric analysis.

X-ray diffraction studies were carried out with nickel-filtered CuKα radiation from an Elliot GX6 rotating anode generator, using cameras with either toroidal mirror [9] or double mirror [10] optics. Samples were held between mylar windows and maintained at constant temperature by a circulating-solvent heating/cooling system.

Samples for differential scanning calorimetry were hermetically sealed in aluminium pans and studied in a scanning calorimeter head adapted to a Dupont 900 Differential Thermal Analyzer. Micellar samples (low ganglioside concentrations) required higher sensitivity, and were run on a Perkin-Elmer DSC-2 scanning calorimeter. Repeated heating and cooling runs were done at 10°C/min between 0°C and 70°C. The sample was then cooled to −50°C to observe the presence or absence of freezable water, and finally heated to 200°C.

Samples were also examined in the light microscope (Zeiss), both by direct light and between crossed polarizers. A heating stage allowed observation of the samples as a function of temperature.

Results

Polarized light microscopy. Over the concentration range 18—30 wt.% H_2O, samples are hard and brittle, and birefringent when observed between crossed polarizers. When water is added, in excess, to the edge of the microscope slide, the samples lose their sharp edges, develop a hazy birefringent texture, and quickly dissolve into an isotropic solution. Samples prepared in the concentration range 30—50 wt.% H_2O are viscous and flow when the cover slip is depressed. These samples are highly birefringent between crossed polarizers, exhibiting middle phase texture. Again, when an excess of water is added to the edge of the sample, the sample forms an isotropic solution. Myelin figures are not observed. When samples are heated and cooled over the temperature range 0—90°C, no changes in viscosity or texture are discernible.

At water contents greater than approximately 60 wt.%, samples are isotropic liquids under crossed polarizers, indicating the presence of a micellar solution, as observed by Gammack [7].

Differential scanning calorimetry. Calorimetric studies of ganglioside/water mixtures over the range 18—30 wt.% H_2O show the existence of two small broad reversible endothermic transitions in the range 0—60°C, and a large enthalpy irreversible broad transition spanning 130—160°C (Fig. 2). The lower temperature transitions have peak maxima at 30°C and 46°C over the concentration range 20—30 wt.% H_2O, and move apart as the water content is increased to 50 wt.% H_2O (Fig. 3). Both transitions become broader with increased water content over this range (Fig. 2, line b). The large enthalpy transition at 130—160°C is probably associated with molecular degradation, since the low temperature transitions (at 30°C and 46°C) are not observed after samples are heated through the high temperature transition and cooled. At water contents greater than 65 wt.% H_2O (micellar solution), two small broad reversible transitions are observed, with peak maxima at 15°C and 39°C.

Below approximately 40 wt.% H_2O, no peak corresponding to the freezing or melting of water is observed. This unfreezable, or bound, water comprises the water of hydration of the ganglioside head groups, and amounts to approximately 60 water molecules per ganglioside molecule, assuming a molecular weight of 1700 for the mixed brain gangliosides (calculated from the composition determined by thin layer chromatography).

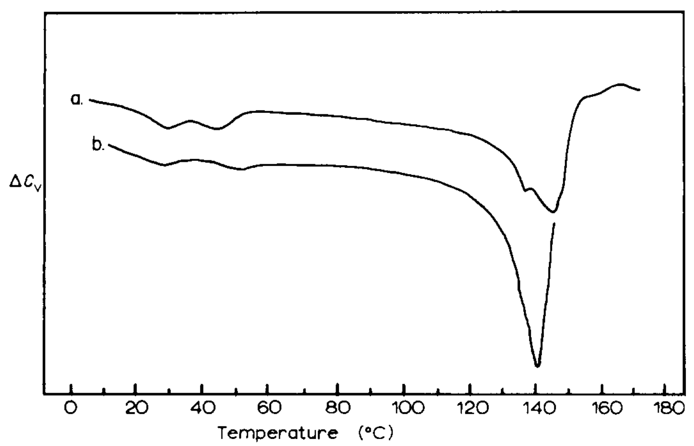


Fig. 2. Differential scanning calorimetry traces (heating runs) of ganglioside/water mixtures. (a) 35.4% H_2O; (b) 40.8% H2O.

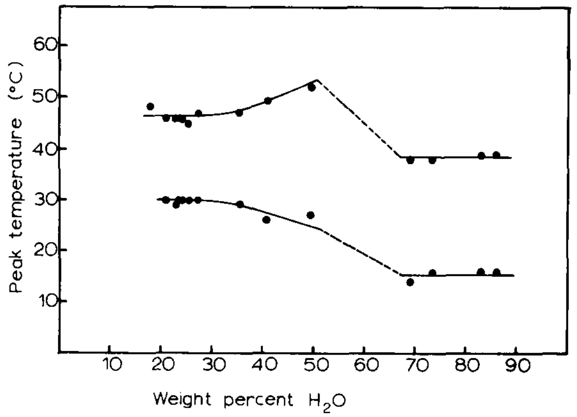


Fig. 3. Calorimetric transition temperatures (peak) as a function of water content.

X-ray diffraction. Over the concentration range 18—50 wt.% H_2O, low angle diffraction lines are observed which index as $1 : 1/\sqrt{3} : 1/\sqrt{7}$. This observation, in conjunction with the observation of middle phase texture in the polarized light microscope, indicates that the most likely structure for mixed brain gangliosides over this concentration range is hexagonal, although the $1/\sqrt{4}$ reflection has not been observed at the water contents studied. Such a structure for gangliosides would consist of hexagonally packed cylinders whose surface comprises the large carbohydrate head groups, and whose interior contains the hydrophobic sphingosine and fatty acyl chains (Fig. 4).

Structural parameters for this hexagonal phase were calculated as described in the Appendix. The total radius of a ganglioside cylinder (Fig. 5), r_G, decreases with increasing water content until it reaches a constant value of 31 Å at 16°C and 28 Å at 55°C. The radius of the "lipid" (non-sugar) core of a ganglioside cylinder, r_L, is 21 Å at 16°C and 19 Å at 55°C. The thickness of the annulus formed by the sugar head groups, r_S, obtained by subtraction, is 10 Å at 16°C and 9 Å at 55°C. The surface area per molecule on the cylinder surface

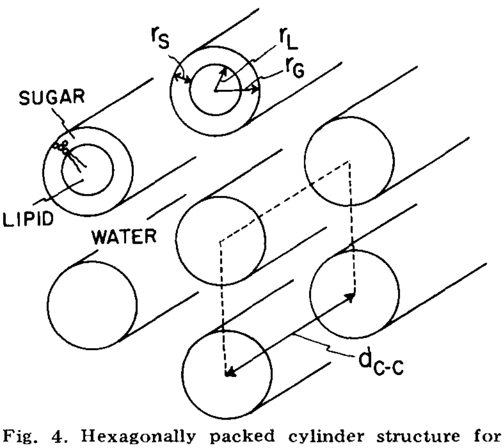
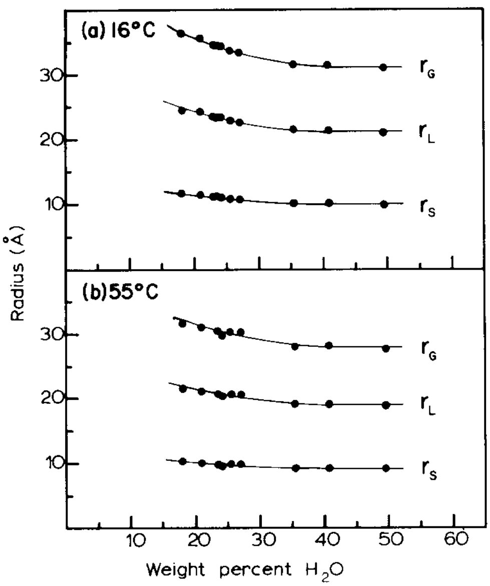


Fig. 4. Hexagonally packed cylinder structure for gangliosides in water ($\%H_2O$ = 18—50%). (After Luzzati, ref. 17).



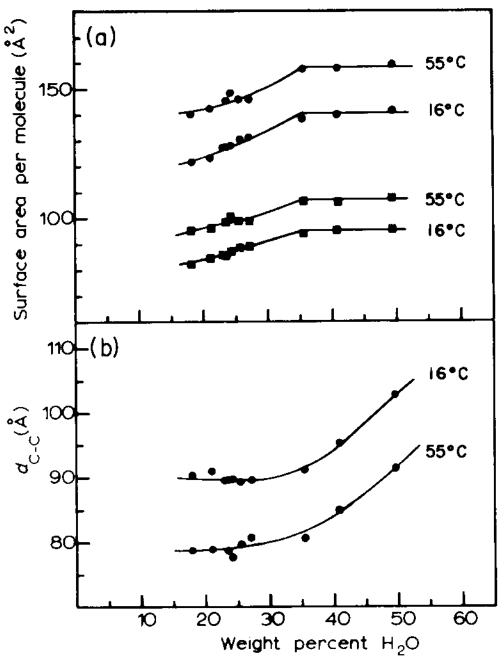


Fig. 5. Cylinder radius as a function of water content. r_G: radius of the entire ganglioside cylinder; r_L: radius of the apolar "lipid" core; r_S: width of the sugar annulus (see Appendix).

Fig. 6. (a) Surface area per molecule on the cylinder surface as a function of water content. ●———●, at outer surface of ganglioside cylinder; ■———■, at "lipid"-sugar interface. (b) Center to center distance between cylinders as a function of water content (see Appendix).

was calculated both at the outer surface of the ganglioside cylinder (S_G) and at the interface between the apolar lipid core and the sugar annulus (S_L). At 55°C, S_G reaches a maximum of 158 Å^2/molecule and S_L reaches 107 Å^2/molecule. At 16°C, S_G and S_L reach maxima of 140 Å^2/molecule and 95 Å^2/molecule, respectively (Fig. 6a). The center-to-center distance, d_{C-C}, between cylinders (Fig. 6b) remains constant until approximately 35% H_2O, and then increases as the water content is increased. The behavior of these parameters as a function of water content is consistent with the choice of a model with the water on the outside of the cylinders and the glycosphingolipid acyl chains on the inside. As the water content is increased past the composition required to hydrate the ganglioside headgroups ($\approx$ 40 wt.% H_2O as determined by differential scanning calorimetry), the model predicts an increase in the center-to-center distance and an invariant radius, as is observed (Figs. 6b and 5, respectively).

At 16°C, for all ganglioside/water mixtures in the hydration range 18—50 wt.% H_2O, a diffuse wide angle diffraction line is present at 4.2 Å. At 55°C, a single diffuse line at 4.5Å is observed at wide angle. Densitometer tracings of the wide angle region are shown in Fig. 7, illustrating the diffuse nature of the wide angle line at both 16°C and 55°C. For a ganglioside/water mixture at 35.4 wt.% H_2O, the wide angle spacing is constant at 4.2 Å over the temperature range 15—35°C, increases gradually to 4.5 Å over the range 35—50°C, and remains constant at 4.5 Å at temperatures greater than 50°C. The structural

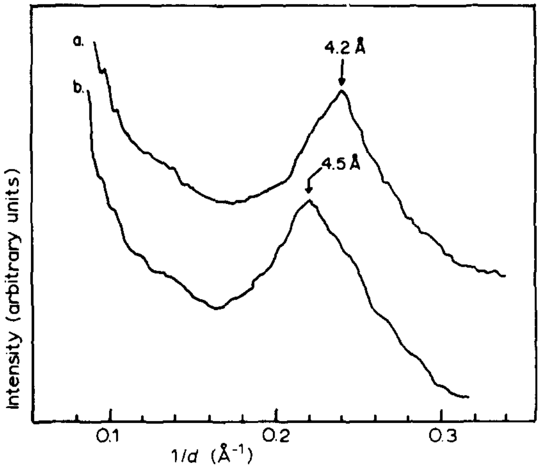


Fig. 7. Densitometer tracings of the wide angle region of the ganglioside/water diffraction pattern (35.4 wt.% H_2O). (a) 16°C; (b) 55°C.

parameters of the hexagonal phase also undergo a discontinuous change over the temperature range 35—50°C. These changes occur over the same temperature range as the calorimetric transition with peak maximum at 46°C.

Discussion

These results indicate that mixed brain gangliosides possess physical properties which are significantly different from those of membrane phospholipids. At low water contents (18—50 wt.% H_2O) these complex glycosphingolipids exhibit a hexagonal mesophase structure of the type illustrated in Fig. 4, while at higher water contents a micellar solution is observed. These observations are similar to those of Abrahamsson et al. [11] on the phase behavior of hydrated psychosine, a monogalactosyl ceramide which has no amide-linked fatty acid. This positively charged glycosphingolipid forms a lamellar phase at water contents less than 48 wt.%, a hexagonal phase in the range 48—66 wt.%, and a micellar solution at higher water contents. Abrahamsson et al. [11] also observed that cerebroside, an uncharged monogalactosyl ceramide, forms a lamellar liquid crystalline phase in water. Sulfatide, which is a sulfated (negative charged) cerebroside, forms lamellar, cubic, and micellar phases with increasing water content [11]. It appears that while cerebroside behaves like lecithin in forming a lamellar phase, the charged glycosphingolipids (psychosine, sulfatide, gangliosides) form other structures. The gangliosides in particular do not form a lamellar structure at any water content greater than 18 wt.%. (Lower water contents were difficult to study due to the hydroscopic nature of gangliosides.) The accumulation of large amounts of any of these charged glycosphingolipids in membranes would probably disrupt the bilayer structure, as has been shown by Hill and Lester [8] for mixtures of gangliosides and phosphatidylcholine.

The amount of water which can be bound by gangliosides in the hexagonal phase (60 mol H_2O/mol ganglioside) is striking when compared with egg lecithin, which has been observed to bind about 10 molecules of water per molecule in the lamellar phase [12,13]. Inspection of molecular models (Fig. 1) reveals that the carbohydrate head groups of gangliosides are enormous when compared with the phosphocholine head group of phosphatidylcholine, emphasizing the potentially unusual characteristics of these complex molecules.

The hexagonal phase of gangliosides exhibits two small broad thermal transitions in the range 20—55°C, similar to those reported for the lamellar phase of bovine brain sphingomyelin [14]. The double transition in natural sphingomyelin has been shown recently [15] to be a complex function of so far undefined parameters, and may not simply be due to a phase separation involving molecules containing two predominant types of amide-linked fatty acids as was originally suggested [14]. The ganglioside thermal transitions are probably due to chain packing rearrangements similar but not identical to the order-disorder transitions of lecithins and sphingomyelins. While the phospholipid order-disorder transition is a change from one lamellar state to another, the low temperature ganglioside transitions involve a change from one hexagonal state to another. Molecular models indicate that a rigid hydrocarbon chain structure for gangliosides in the hexagonal phase is sterically unlikely, and, in fact, no rigid-chain hexagonal phase has been reported in the literature. Wide-angle X-ray diffraction data (Fig. 7) indicate that, both above and below the thermal transitions, the hydrocarbon chain portions of the gangliosides are disordered, and so these thermal transitions are related to a change from one disordered state to another possibly more disordered state.

In Tay-Sachs disease, Sandhoff's variant, and generalized gangliosidosis, gangliosides accumulate in membrane-enclosed multilayered lamellar bodies, along with phospholipid and cholesterol [16]. It is possible that the cell reacts to the accumulation of gangliosides with increased phospholipid and cholesterol synthesis in an attempt to sequester the gangliosides in an intracellular lamellar structure, thus limiting their accumulation in the plasma membrane and other membranes. Another possibility is that the glycosyl hydrolases involved in ganglioside degradation in vivo may act only on gangliosides in a bilayer structure, and so gangliosides must be combined with phospholipid and cholesterol to present the proper orientation of the substrate to the enzyme. When a particular glycosyl hydrolase is missing, as in the various gangliosidoses, larger multilamellar aggregates accumulate. The study of the physical properties of the gangliosides alone, and then in combination with other membrane lipids is a prerequisite for the detailed understanding of these complex phenomena.

Appendix

The structural parameters of the hexagonal phase are determined using the relationships described by Luzzati [17]. We have calculated these parameters for both the ganglioside molecule as a whole, and for the "lipid" (non-sugar) core formed by the apolar portion of the ganglioside molecule.

The volume fraction ganglioside is defined by,

$$\phi_G = \frac{\bar{v}_G C_G}{\bar{v}_G C_G + \bar{v}_W C_W}$$

where C_G and C_W are the weight fractions ganglioside and water, respectively. The partial specific volume for ganglioside ($\bar{v}_G$) is 0.78 cm^3/g [8] and that for water ($\bar{v}_W$) is 1.00 cm^3/g. The radius of the ganglioside cylinders is,

$$r_G = \left(\frac{\sigma\phi_G}{\pi}\right)^{1/2}$$

where σ is the area of the primitive two-dimensional unit cell,

$$\sigma = \frac{\sqrt{3}}{2} d_{01}^2$$

where d_{01} is the d-spacing corresponding to the first-order reflection of the hexagonal diffraction. The mean area per ganglioside molecule at the ganglioside-water interface is,

$$S_G = \frac{2\pi r_G}{N_G}$$

where,

$$N_G = \frac{\sigma\phi_G N 10^{-24}}{M_G \bar{v}_G}$$

N if Avogadro's number; M_G is the average molecular weight of bovine brain ganglioside, 1700 g/mol (calculated from the composition by thin layer chromatography).

The partial specific volume of the "lipid" core of the ganglioside cylinders (fatty acid plus sphingosine) was calculated as follows. If we take the average M_G of mixed brain gangliosides to be 1700 g/mol and the average M of the non-sugar part of a ganglioside to be 580 g/mol (M of stearic acid plus sphingosine), then the ganglioside molecule is 34% "lipid" and 66% sugar. Taking the average $\bar{v}$ for a variety of sugars ($\bar{v}_S$) to be 0.64 cm^3/g (CRC Handbook of Chemistry and Physics), we can obtain an approximate partial specific volume for the lipid portion of ganglioside ($\bar{v}_L$) from,

$$\bar{v}_G = 0.34\,\bar{v}_L + 0.66\,\bar{v}_S$$

which gives a $\bar{v}_L$ of 1.05 cm^3/g. The partial specific volume of stearic acid is 1.06 cm^3/g (CRC Handbook of Chemistry and Physics), which agrees with our derived $\bar{v}_L$.

Since the ganglioside molecule is 34% "lipid",

$$C_L = 0.34\ C_G$$

and,

$$\frac{\phi_L}{\phi_G} = 0.34\,\frac{\bar{v}_L}{\bar{v}_G}$$

giving,

$$\phi_L = 0.458\ \phi_G$$

for the volume fraction of "lipid". The radius of the "lipid" core of the ganglioside cylinders is,

$$r_L = \left(\frac{\sigma\phi_L}{\pi}\right)^{1/2}$$

The width of the annulus formed by the sugar portion of the ganglioside molecules is obtained by subtraction:

$$r_s = r_G - r_L$$

The mean area per molecule at the "lipid"-sugar interface is,

$$S_L = \frac{2\pi r_L}{N_L}$$

where,

$$N_L = \frac{\sigma\phi_L N 10^{-24}}{M_L\bar{v}_L} = N_G$$

The center to center distance between cylinders was calculated from,

$$d_{C\text{—}C} = \frac{2}{\sqrt{3}}\, d_{01}$$

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