Surface Behaviour of Gangliosides and Related Glycosphingolipids

By BRUNO MAGGIO, FEDERICO A. CUMAR and RANWEL CAPUTTO
Departamento de Química Biológica, Facultad de Ciencias Químicas,
Universidad Nacional de Córdoba, Córdoba, Argentina
(Received 25 October 1977)

1. The surface behaviour of six different gangliosides and eight chemically related glycosphingolipids was investigated in monolayers at the air–water interface. 2. Mono-, di-, tri- and tetra-hexosylceramides had force-area isotherms showing similar limiting molecular areas on 145 mM-NaCl, pH 5.6. The increasing number of negatively charged sialosyl residues in mono-, di- and tri-sialogangliosides induced a progressive increase in the liquid-expanded character of the films and in the limiting area occupied per molecule, owing to electrostatic repulsions. When the ganglioside monolayers were spread on subphases at pH 1.2, the limiting area per molecule was similar to that found for neutral glycosphingolipids. 3. The monolayer collapse pressure at pH 5.6 increased with the number of uncharged carbohydrate units up to when the polar head group contained 3–4 residues. For gangliosides the collapse pressures were lower and decreased from mono- to tri-sialogangliosides. Ganglioside monolayers on subphases at pH 1.2 showed increases in their collapse pressure. 4. The glycosphingolipid monolayers studied had various surface potentials according to the complexity of the polar head group of the lipid. Attempts to calculate the dipolar contributions to the surface potential from each carbohydrate residue suggest that the second and third sialosyl residues in di- and tri-sialogangliosides contributed with a vertical dipole moment opposite to that of the first sialosyl residue.

There is evidence that gangliosides are available in cell and membrane surfaces as substrates of neuraminidase (Maccioni et al., 1974) and as receptors of hormones and toxins (for review see Fishman & Brady, 1976). Also, they have been shown to increase the glucose permeability of liposomes in the presence of neurotransmitters (Maggio et al., 1977). However, precise knowledge of the location and role of this group of lipids in membrane organization probably requires elucidation of their surface properties.

Lipid monolayers are a suitable experimental system for the study of the surface behaviour of amphipathic molecules in an oriented molecular array. This system has been used in the studies reported here on gangliosides and a series of related glycosphingolipids to obtain information on the influence that the complexity of their polar head groups may have on their interfacial properties.

Materials and Methods

The different lipids used and their abbreviations are listed in Table 1. GalCer and GlcCer were obtained by the method of Radin & Brown (1960) from bovine brain and spleen from a patient with Gaucher’s disease respectively. GalSpd and GlcSpd were obtained from GalCer and GlcCer respectively as described previously (Cumar et al., 1968). Stearoyl-Spd was prepared from GalSpd by a combination of the procedures of Kopaczyk & Radin (1965) and Carter et al. (1961). LacCer, Gg3Cer and Gg4Cer were obtained by partial hydrolysis of total brain gangliosides (Cumar et al., 1968). Ganglioside GM1 was isolated from horse erythrocytes, ganglioside GM2 was obtained from the brain of a patient with Tay–Sachs disease and ganglioside GD3 from bovine retina (Cumar et al., 1971). Gangliosides GM1, GD1, and GT1 were isolated from a mixture of bovine brain gangliosides (Mestrallet et al., 1977). T.I.C. of each purified lipid in amounts at least 10 times that required for detection showed no contaminant spots after charring the plate with 50% (v/v) H2SO4. LacSpd and NaCl were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The NaCl was roasted at 400–500°C for 5h. Solvents were redistilled and further purified through alumina. Water was double-distilled over alkaline KMnO4 in an all-glass apparatus.

The equipment used to measure the properties of the monolayers was similar to that used by Maggio & Lucy (1975, 1976). The Teflon trough had an area of 92 cm² and was 0.8 cm deep; it was enclosed in a Plexiglass box and surrounded by a Faraday cage. Surface pressure, surface area and surface potential were measured simultaneously at a constant rate of compression (7.1 cm²/min) with a Teflon barrier. Surface pressures were measured with a platinumized-platinum dipping wire (diameter 0.55 mm) suspended
from a LM 600 Beckman electronic microbalance (Beckman-R1C, Glenrothes, Scotland, U.K.). Measurements of surface potential were made with a Beckman Zeromatic SS-3 pH-meter connected to an air-ionizing electrode of $^{24}$Am suspended at 5–7 mm from the air–water interface and to a calomel reference electrode connected to the subphase through a salt bridge. The outputs from the electric balance and the millivolt-meter were continuously recorded on the ordinate of a two-channel W+W Tarkan recorder (W+W electronic Inc., Basel, Switzerland), whose abscissa recorded the surface area. Measurements were made at $20 \pm 1 ^\circ C$ on subphases of unbuffered 145 mm-NaCl at a specified pH adjusted with HCl or NaOH. The different glycosphingolipids were spread from solutions in chloroform/methanol (2:1, v/v) or in chloroform/methanol (2:1, v/v) containing 5% water. Isotherms were measured 1–2 min after spreading the glycolipid. The absence of surface-active impurities from the solvents or the subphase was measured before spreading the films by the changes in surface pressure and surface potential that occurred on decreasing the surface area to 10% of its initial value. These changes were less than 0.1 mN·m$^{-1}$ or 5 mV for the subphase alone or with spreading solvents. The surface pressure–surface potential–area curves were obtained in about 10 min. The values reported are averages of duplicate or triplicate runs. Reproducibility for the different isotherms was within ±0.02 nm$^2$ for surface areas, within ±1 mN·m$^{-1}$ for surface pressures and within ±5 mV for surface potentials. Except for Cer at surface pressures under 5 mN·m$^{-1}$ and for LacSpd, which was somewhat soluble in the subphase, the different compounds did not show signs of instability or solubility; their isotherms were reproduced if the films were allowed to expand and were compressed again.

**Results and Discussion**

**Area per molecule and surface pressure**

The surface pressure–area curves on 145 mm-NaCl, pH 5.6, for sphingolipids of increasing complexity from Cer to ganglioside GT$_1$ are shown in Fig. 1(a). The area–area isotherms for Cer, GalCer and GlcCer were more condensed than those for the rest of the series; they were practically identical among themselves except at pressures below 15 mN·m$^{-1}$, where GalCer and GlcCer showed a more expanded isotherm. These data are in general agreement with those reported by Quinn & Sherman (1971), Stoffel et al. (1974), Maggio & Lucy (1975) and Oldani et al. (1975), although precise comparisons are not possible, since the experimental conditions were not identical.

As the number of carbohydrate residues in the molecule was increased from LacCer to Gg$_2$Cer and Gg$_3$Cer, the curves shifted to greater values of area per molecule at a given surface pressure below...
collapse. However, in every case the limiting area per molecule that was obtained just before collapse was within 0.07 nm² of the area for Cer. This limiting area of 0.38–0.45 nm² was approximately equal to 0.44 nm² shown by dipalmitoyl phosphatidylcholine (1,2-hexadecanoyl-sn-glycero-3-phosphocholine) (Maggio & Lucy, 1976) and is to be expected for two closely packed saturated hydrocarbon chains. These results suggest that at high pressures the carbohydrate residues are dipping into the subphase and thus fail to contribute appreciably to the cross-sectional area of the ceramide moiety of the whole molecule, whereas some contribution from the hydrophilic chain was apparent at lower pressures.

The introduction of sialosyl groups into the molecule caused a marked increase in the limiting molecular area, and the monolayers became more expanded (Fig. 1a). Ganglioside GT₁, which showed a very expanded type of isotherm, behaved as a gaseous film at the lowest surface pressure (cf. Gaines, 1966).

The intensification of the liquid character of the films of gangliosides with increasing number of sialosyl residues was also evidenced by the increase in surface compressibility of the monolayers (Fig. 1a, inset; cf. Sears & Stark, 1973).

The monosialogangliosides GM₁, GM₂ and GM₃ had isotherms showing similar areas, within 0.10 nm², for a given surface pressure; this was also apparent for ganglioside GD₂ and GD₁a, the two disialogangliosides studied. This and the greater areas per molecule occupied by gangliosides with an increasing number of sialosyl groups suggest that the expansion of the films was the consequence of an increased electrostatic repulsion between the negative charges. Experiments carried out at pH 1.2 (see below) lent support to this conclusion. Curatolo et al. (1977) have determined by X-ray diffraction the average molecular area for a mixture of bovine gangliosides in a hydrated hexagonal phase. Although a direct comparison is difficult, their values are in general

---

**Fig. 1. Surface behaviour of glycosphingolipids**

The surface pressure–area (a) and the surface potential–area (b) curves were determined at pH 5.6 for GlcSpd and GalSpd (▲), LacSpd (●), Cer (○), GlcCer and GalCer (△), LacCer (□), Gg₃Cer (◇), Gg₄Cer (▽), GM₃ (■), GM₂ (●), GM₁ (◇), GD₁a (▽), GD₃ (□) and GT₁ (●). Gg₃Cer and GM₃ show a transition from a liquid-expanded to a liquid-condensed state at about 5 mN·m⁻¹ (0.79 nm²) and 15 mN·m⁻¹ (0.95 nm²) respectively. The inset shows the surface compressibility of the films at 30 mN·m⁻¹ for the lipids indicated.
agreement with ours if the molar fraction of each ganglioside in the mixture is taken into account.

At pH 5.6 very expanded isotherms were also observed for GalSpd, GlaSpd and LacSpd with respect to the isotherms for GalCer, GlaCer and LacCer. Since in each of the former compounds a free amino group is present, we assumed that a positive charge owing to the protonation of the long-chain amine caused the electrostatic repulsion in those cases.

The collapse pressure of a film is related to effects such as the cohesion between film molecules and the affinity of their polar head groups for the aqueous subphase (Gaines, 1966). The collapse pressures for the compounds studied (Fig. 2a) increased with the number of carbohydrate units up to three or four uncharged residues. Introduction of negatively charged sialosyl groups caused a decrease of the collapse pressure of the monolayer. Thus a drastic decrease in the collapse pressure for ganglioside GD3 compared with that of ganglioside GM3 was observed at pH 5.6, that for ganglioside GD3 being similar to that of ganglioside GD1a. Conversely, the removal of a sialosyl group from ganglioside GM1 to give Gg4Cer or from GM2 to give Gg3Cer caused an increase in the pressure required for the film to collapse. From the foregoing results it appeared that three to four uncharged carbohydrate residues attached to a pair of hydrophobic chains conferred optimal stability on the monolayer arrangement, probably by providing a more suitable length of the hydrophilic with respect to the hydrophobic portion. Removal of a fatty acyl chain as in GalSpd, GlaSpd or LacSpd, or introduction of a charged residue as in gangliosides, or removal of the carbohydrate chain as in ceramide, unbalanced the interfacial forces, and the surface pressure required to modify the monolayer arrangement decreased correspondingly.

Monolayers of GlaSpd, LacSpd, Gg4Cer, Gg3Cer, and gangliosides GM3, GM2, GM1, GD1a, GD3 and GT were also studied on 145 mM NaCl at pH 1.2 and 10.4. Fig. 2(b) summarizes the data for molecular areas at two different surface pressures obtained at these pH values. At pH 1.2 the limiting cross-sectional area of the different gangliosides showed values within 0.07 nm² of those for Gg4Cer or Gg3Cer. Since the limiting cross-sectional areas for these last compounds at pH 1.2 were unchanged with respect to pH 5.6, these results showed that protonation of the negative charge of the sialosyl group (pKₐ = 2.6) allowed a closer packing of the gangliosides by diminishing electrostatic repulsions. Thus in closely packed uncharged gangliosides the carbohydrate chain, possessing between three and seven units, does not produce an increase of the area per molecule, whereas at low pressures some contribution is observed (Fig. 2b).

Values obtained for gangliosides, Gg4Cer and Gg3Cer on subphases at pH 10.4 were practically the same at all pressures as those found at pH 5.6. For GlaSpd and LacSpd the isotherms at pH 1.2 were similar to those at pH 5.6, but showed lower areas per molecule at all pressures at pH 10.4. This was probably due to the deprotonation of the sialosyl moiety, thus decreasing electrostatic repulsions.

Besides decreasing areas per molecule the elimination of the charges induced increases in the collapse pressures of the monolayers (Fig. 2a), probably
owing to an increase in the cohesive forces between the molecules allowing a closer packing. However, it was apparent that as the number of uncharged carbohydrate residues exceeded three to four units the collapse pressure decreased.

Surface potential

The surface potential–area curves for neutral sphingolipids and gangliosides on 145 mm-NaCl at pH 5.6 are shown in Fig. 1(b). For neutral glycosphingolipids, increased complexity in their polar head groups caused marked changes in the surface potential–area curves with respect to the relatively small differences in their molecular packing at high pressures. For gangliosides the surface potential–area isotherms are less readily comparable, because of the differences in the area occupied per molecule.

Average surface potentials per molecule, \( \Delta V/n \), where \( n \) is the number of molecules per cm\(^2\), calculated from \( \Delta V/n = 10^{-14} \times \Delta V/A \) (mV·nm\(^2\)·molecule\(^{-1}\)) (Shah, 1970) are shown in Fig. 3(a). The surface potential contributed per molecule in the closest-packed state in the monolayer was higher for ceramide than the comparable values for GlcCer or LacCer. Removal of the fatty acyl chain from these latter compounds lowered still further the surface potential contributed per molecule. Values of \( \Delta V/n \) for LacCer, Gg5Cer, Gg4Cer and gangliosides GM\(_3\), GM\(_2\) and GM\(_1\) were similar. Gangliosides GD\(_{14}\) and GD\(_3\) showed similar values, but these were higher than those of the monosialogangliosides and an even higher value was found for ganglioside GT\(_1\) at pH 5.6.

The above data showed that the surface potential per molecule of gangliosides containing two or three sialosyl groups, such as ganglioside GD\(_{14}\), GD\(_3\) and GT\(_1\), at pH 5.6 was significantly higher than those containing just one, whereas little change was caused by incorporation of the first sialosyl groups into asialo derivatives. However, in attempting to interpret the differences in \( \Delta V/n \) for this series of compounds it should be kept in mind that this parameter has a different meaning for uncharged and charged molecules, since in the latter an ionic double layer is contributing to the total surface potential in the monolayer.

Assuming a value of unity for the dielectric constant, the surface potential of a monolayer can be expressed (cf. Gaines, 1966) as:

\[
\Delta V = \frac{0.12\pi}{A} (\mu_+ + \psi_0) \tag{1}
\]

where \( A \) is the molecular area in nm\(^2\); \( \psi_0 \) is the potential difference of the ionic double layer in ionized monolayers (for uncharged lipids \( \psi_0 = 0 \); \( \mu_+ \) is the overall dipole moment in the direction perpendicular to the interface resultant from the contribution of the dipole moments \( \mu_1 \) from the orientated water molecules, \( \mu_2 \) from the polar head group and \( \mu_3 \) from the hydrocarbon chains (Davies & Rideal, 1963). Thus \( \Delta V \) is a complex quantity resulting from several independent and not readily separable contributions. However, since the chemical structure of the glycosphingolipids follows a well-established pattern, comparisons between them are possible. For most of these compounds the hydrophobic portion of the molecule was, on average, identical (Yohe et al., 1976), especially in the case of some that were prepared from other components of the series. Therefore the \( \mu_3 \) contribution was assumed to be equal throughout. In addition the contribution of the polar head groups of the different compounds can in every case be related to a compound that has an identical polar head except for the lack of a carbo-
hydrate unit. Consequently the hydration shell of the common part of the carbohydrate chain of two compounds so related were considered to be grossly equal and the difference in the resultant \( \mu_{\perp} \) was attributed to the added carbohydrate unit.

Assuming that \( \psi_0 \) can be calculated as a first approximation from the Gouy equation (Davies & Rideal, 1963; Gaines, 1966) it can be discounted. At 20°C

\[
\psi_0 (mV) = 50.4 \sinh^{-1}(1.34 \times 10^4 / A c)
\]

where \( A \) is the area per monolayer ion in nm² and \( c \) the molar concentration of uni-univalent electrolyte in the subphase.

Fig. 3(b) shows the value of the overall resultant vertical dipole moment contributed by the molecule \( (\mu_{\perp}) \) at the limiting molecular area, calculated from eqn. (1) after the potential contribution by the ionic double layer was discounted. The monosialogangliosides GM₂, GM₁ and GM₀ showed similar values for \( \mu_{\perp} \) at pH5.6 (between 50 to 120 mD). Unlike the values for \( \Delta V/n \), the values for \( \mu_{\perp} \) after discounting the contribution of a predominantly positive ionic layer were considerably lower in the monosialogangliosides than in the neutral glycosphingolipids, the values for which were greater than 250mD. For gangliosides GD₂ and GD₁₈, \( \mu_{\perp} \) was similar to those for neutral glycosphingolipids, whereas for ganglioside GT₁ it was higher and similar to that for Cer (greater than 500mD). These results indicate that in gangliosides the contribution by the second and third sialosyl residues to the perpendicular electric field was different from that of the first sialosyl group. Since the surface potential is positive the overall resultant \( \mu_{\perp} \) of the molecule is considered as a vector having its air end positive (Gaines, 1966). The first sialosyl group is thought to introduce a net dipole moment in the opposite direction, thus causing a lowering in \( \mu_{\perp} \), whereas the second and third sialosyl residues apparently introduce resultant dipoles of similar magnitude to the first but having a positive air end. The data for gangliosides GM₂, GM₁, GD₁₈ and GT₁ at pH1.2 (Fig. 3a) showed \( \Delta V/n \) values that were similar for all of them and to those for neutral glycosphingolipids; a comparable pattern was found for \( \mu_{\perp} \) (Fig. 3b). The abolition of the charge on the sialosyl residue in gangliosides GM₃, GM₂ and GM₁ induced an increase in the overall vertical dipole, suggesting again that the contribution to \( \mu_{\perp} \) of the negative charge on this residue is in an opposite direction to the resultant dipole moment of the molecule. For gangliosides GD₁₈ and GD₂, practically no change in \( \mu_{\perp} \) was found on discharge of their two sialosyl groups. This can be explained provided that one contributed in the same and the other in the reverse direction of the molecular \( \mu_{\perp} \). For ganglioside GT₁, a decrease in the resultant \( \mu_{\perp} \) was found at pH1.2 compared with pH5.6, as would be expected if two vectors positive upward and one in the opposite direction are discharged at pH1.2. At pH10.4 the molecular \( \mu_{\perp} \) values for gangliosides were practically the same as at pH5.6.

Elimination of the fatty acyl group from GlcCer and LacCer to produce GlcSpd and LacSpd caused a decrease in the \( \Delta V/n \) values at pH5.6 or 1.2 (Fig. 3a). GlcSpd and LacSpd were positively charged at those pH values, and an ionic double layer, predominantly negative, was contributing to the surface potential. When this value was discounted the overall resultant \( \mu_{\perp} \) showed values almost identical with those of GlcCer and LacCer at pH5.6 and 1.2 (Fig. 3b). These results, however, should not be interpreted as if the fatty acyl group has no contribution to the resultant dipole moment of GlcCer and LacCer. The vertical-dipole-moment contribution of the positively charged amino group in GlcSpd and LacSpd is in the same direction as that of the whole molecule and that of the fatty acyl residues, having a similar magnitude to the latter. This was concluded from the decrease in the resultant \( \mu_{\perp} \) for GlcSpd and LacSpd uncharged at pH10.4.

A diagrammatic representation of the contributions to \( \mu_{\perp} \) of the different components of glyco-
sphingolipids as concluded from the results in the present work is given in Fig. 4. In this illustration, calculated from the values in Fig. 3(b), the differences in \( \mu_i \) introduced by changes in ionization of ionizable groups are also included.

Study of the surface behaviour of the glycosphingolipids reveals that this series of closely related compounds shows, however, great differences in their molecular packing and surface potential contributions owing to the ample possibilities of variation in their polar head groups. The different surface behaviour of these glycosphingolipids and the influence that the state of ionization has on their molecular arrangement and electrical field at an interface may help to explain some of the ganglioside functions. In this connection, the orientation and sign of molecular dipoles at the surface of a lipid bilayer may be more important than formal charges in regulating ion permeabilities (Bangham, 1968). It has recently been proposed that induction of permeability changes may be a common feature of the action of different hormones and toxins, for which gangliosides are postulated as receptors (Grollman et al., 1977).

This work was supported in part by grants from the Organización de los Estados Americanos, Fundación Lucio Cherny and the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. B. M. is a Career Investigator of the last institution. We are grateful to Dr. H. J. F. Maccioni, Dr. J. A. Curtino and Dr. M. G. Mestrallet for gifts of some glycosphingolipids. We thank Mr. H. Di Vito for invaluable help in constructing part of the equipment used in these studies.

References

Kopacz, K. C. & Radin, N. S. (1965) J. Lipid Res. 6, 140–145