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HEADGROUP BEHAVIOUR OF AN UNCHARGED COMPLEX GLYCOLIPID

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A globoside spin labelled on the terminal sugar residue has been synthesized, and employed in model membranes to study headgroup behaviour of complex uncharged glycolipids. The labelled headgroup demonstrated a high degree of motional freedom limited to the aqueous region of the interface between lipid bilayer and surrounding medium. This observation was unaltered by the presence of a dense, tightly-bound surface layer of peripheral proteins or polysaccharide—which might be expected to reproduce conditions present at a cell surface. Headgroup dynamics were only very modestly correlated with the physical state (i.e., fluidity) of the membrane itself. In spite of the absence of charged sugar residues in globoside, the aspects of its headgroup behaviour monitored here we found to be similar to those of oligosaccharide chains on gangliosides and several sialic acid-rich glycoproteins.

Introduction

Because of the widely accepted view that cell surface structures are importantly involved in recognition, adhesion and migration, considerable attention is currently being focussed on their arrangement and dynamics within the membrane. One set of surface components which deserves careful study is the family of lipids known as glycosphingolipids. While their ceramide backbones are an integral part of the lipid bilayer hydrophobic region, their oligosaccharide headgroups form part of the glycocalyx and contribute importantly to the tissue and cell cycle-specific surface 'fingerprint' [1–3]. The purpose of this article is to consider the behaviour of uncharged glycosphingolipid headgroups as it relates to other cell surface structures and to the hydrophobic membrane interior. This is an area which has already been investigated for the negative-charged headgroups of gangliosides by this laboratory and several others (Refs. 4–10 and references therein). A fairly consistent picture seems to be emerging in the latter case: one in which ceramide backbones behave like phospholipids while oligossacharide headgroups wag freely at the lipid/water interface. The question of charged vs. neutral sugar headgroups has been addressed very nicely by Maggio et al. [9]. Although these authors caution that their experiments with monolayers may not be trivially extrapolated to bilayers, they have put forward a convincing argument for the significance of charged sugars in determining surface energies and interactions.

The approach our laboratory has taken to the study of membrane receptors has been to isolate them for assembly into model membranes where one may focus on some aspect of interest. By specifically attaching a spectroscopic probe such as the nitroxide spin label to given membrane components it is possible to monitor their molecular environment and motional properties. The neutral glycolipid chosen for study in this work is one of the globoside family with the formula, GalNAc

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(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)GlcCer. This particular globoside is the major neutral glycolipid of the human erythrocyte membrane [11]. But for their lack of (negative-charged) *N*-acetylneuraminic acid residues, globosides are very similar to gangliosides. Hence we have attempted to draw comparisons between these two families in order to gauge the significance of this feature to head-group behaviour.

Materials and Methods

Sources of phospholipids and glycophorin, as well as methods of lipid bilayer preparation were as described previously [7,12]. Globoside was isolated from porcine red blood cells according to the method of Hakomori and Siddiqui [13], except that the crude sphingolipid extract was run on a silicic acid column (BioSil A, 3 × 70 cm eluted with 1 litre $CH_3OH/CHCl_3$ (1 : 9, v/v) and 1.5 litre $CH_3OH/CHCl_3$ (2 : 8, v/v), followed by a gradient of 2 : 8–4 : 6 (v/v)). The globoside fraction was further purified on a small silicic acid column eluting with $CHCl_3/CH_3OH/NH_4OH$ (60 : 40 : 4, v/v). It was pure as judged by thin-layer chromatography (Stahl, silica gel GF_{254}).

Human intestinal mucin was the kind gift of Dr. Janet Forstner, Hospital for Sick Children, Toronto. Stearic acid was from Serdary Research, London, Ontario. Galactose oxidase, globulin-free bovine serum albumin, Dextran (average M_r 90000), and *N*-hydroxysuccinimide were all from Sigma. Dicyclohexylcarbodiimide was from Eastman Kodak. Free fatty acids were conjugated covalently to bovine serum albumin and 90000 M_r Dextran by the general methods of Lapidot et al. [14] and Wolf et al. [15], respectively.

Spin labelling

Globoside was spin labelled on the terminal GalNAc (Fig. 1) by a general method used by us previously for glycoproteins [12]. 25 mg globoside was dissolved in 2 ml 50/50 0.1 M phosphate buffer and freshly distilled tetrahydrofuran. To this 0.6 mg galactose oxidase was added and the mixture was stirred at room temperature for 72 h. This was then cooled to 4°C and 20 mg 4-amino-2,2,4,4-tetramethylpiperidine-1-oxyl in 4 ml 0.2 M Na_2CO_3 buffer, pH 9.4, was added and stirred for 2 h. 1 ml 0.6 M $NaBH_4$/0.01 M NaOH was finally added to stabilize the Schiff base and to reduce any remaining aldehyde groups. The tetrahydrofuran was evaporated under a stream of nitrogen gas and the remaining globoside suspension was dialysed against Na_2CO_3 buffer for 2.5 h followed by dialysis against distilled water for a further 24 h. The globoside mixture was dried under vacuum and then dissolved in a small volume of 300 mM dodecyltrimethylammonium bromide and dialyzed extensively against distilled water in order to ensure removal of any residual unreacted spin label.

Intestinal mucins were spin labelled on terminal *N*-acetylneuraminic acid or Gal/GalNAc residues as described previously for the integral membrane glycoprotein, glycophorin [12] and bovine submaxillary mucin [16]. Samples of the labelled material were concentrated to various degrees by vacuum dialysis at 4°C until gelation occurred. Asialoglycophorin was prepared and spin labelled as described earlier [12].

Results and Discussion

Purified globoside was spin labelled by selectively attaching a nitroxide-containing ring to the terminal GalNAc residue (Fig. 1). The procedure involved enzymatic oxidation followed by condensation of the activated terminal sugar with an amine-bearing spin label and subsequent stabilization of the resultant Schiff's base by borohydride reduction (see Materials and Methods). We have previously discussed the fact that spin labels attached to oligosaccharide chains in this fashion seem to adequately mimic the motions of sugar residues [4,7,12], and that where oligosaccharide dynamics have been compared as monitored by NMR spectroscopy and EPR spectroscopy [17] the agreement is good.

EPR spectra of labelled globoside in model membranes invariably consisted of three sharp lines. From such spectra the spin label correlation time, τ_c, may be calculated using peak height ratios and linewidth measurements [18]. This parameter is inversely related to spin label (and hence head group sugar) freedom of motion. Another useful spectral feature is the hyperfine splitting, a_N (the distance between spectral lines), which is related to

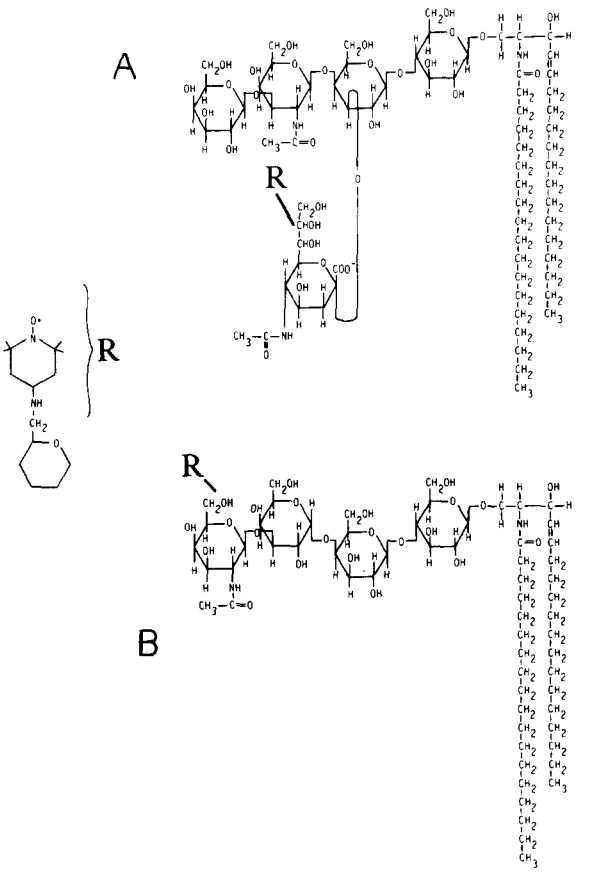

Fig. 1. Site of attachment of spin label, R, to glycolipid headgroups: (A) a typical ganglioside with indicated labelling on C_6 or C_7 of *N*-acetylneuraminic acid; (B) globoside with indicated labelling on C_6 of GalNAc. Insert at left shows mode of spin label liganding.

the polarity of the spin label environment [19,20]. These parameters have been recorded in Table I for spin labelled globoside in a variety of situations. For comparison, values obtained with comparably labelled gangliosides have been included. It is readily apparent that the globoside and ganglioside headgroup motional characteristics reflected in the table are basically very similar. The measured values of headgroup spin label correlation time are typical of relatively unrestricted motion for both glycolipid families. It is also clear that the values of a_N for the headgroup of this particular complex neutral glycolipid suggest no more tendency to interact with membrane hydrophobic regions than do those of the charged gangliosides: the polarity sampled is never detectably less than that of water in either case.

Temperature effects

An indication of how carbohydrate head group behaviour relates to surrounding lipids may also be obtained from temperature studies of the headgroup motional freedom parameter, log $1/\tau_c$. Fig. 2A and B are plots of this variable as a function of $1/T$ for spin labelled globoside at low concentration in bilayers of several pure phospholipids. Such bilayers are well known to exhibit sharp fluid/gel phase transitions at characteristic temperatures-that is they have 'melting points' at which the acyl chains cooperatively and reversibly convert from a rigid crystalline matrix to a highly mobile liquid crystal form (reviewed in Ref. 21). In the case of dimyristoyl and dipalmitoyl phosphatidylcholine these temperatures are 23 and 41.5°C, respectively [22,23]; and the amounts of globoside incorporated were not sufficient to appreciably alter the host phase transition temperature (for instance see Fig. 2C). One would probably expect that the dramatic changes brought about in the phospholipid matrix by varying the temperature through the region of a phase transition would be reflected in carbohydrate head group dynamics. Indeed there are measurable breaks in the curves describing headgroup spin label mobility under these circumstances. However, the inflections observed occur at temperatures below the matrix phase transition temperature, as we reported previously for gangliosides [7]. One possible explanation of this observation is that the major inflection in each case simply occurs in association with the host lipid pretransition (Ref. 24 and references therein) and that the main transition (associated with cooperative melting to the acyl chains) has almost no influence on the oligosaccharide headgroup. This would seem a reasonable suggestion since the pretransition phenomenon may be involved with the phospholipid headgroup region. However, in the case of dimyristoyl phosphatidylcholine the major curve inflection at 19.5°C does not fall within the range 7–14°C corresponding to reported values of the pretransition [24]. Also the observed inflections tend to be several degrees lower in the same host matrix when the experiment is performed with labelled gangliosides

TABLE I

Typical spectral data (spin label correlation time, τ_c and hyperfine splitting, a_N) for headgroup labelled globoside and bovine brain ganglioside under various conditions. All samples were prepared in Hepes-buffered isotonic saline, pH 7.4/2 mM $MgCl_2/CaCl_2$. Values of a_N for the (free) nitroxide spin label used in labelling glycolipids are 16.8 G in buffer and 14.5 G in hexane. See Fig. 1 for label location in glycolipid headgroup.

Conditions	Globoside		Ganglioside	
	τ_c ($\pm 0.5 \cdot 10^{-10}$ S)	a_N (± 0.1 G)	τ_c ($\pm 0.5 \cdot 10^{-10}$ S)	a_N (± 0.1 G)
3 mol% in egg phosphatidylcholine	13.1	16.8	13.8	16.8
3 mol% in dimyristoyl phosphatidylcholine	14.1	16.8	13.6	16.8
3 mol% in dipalmitoyl phosphatidylcholine	15.4	16.8	17.1	16.8
10 mol% in globoside micelles	23.4	—	25.3	—
10 mol% in ganglioside micelles	44.0	—	29.5	—

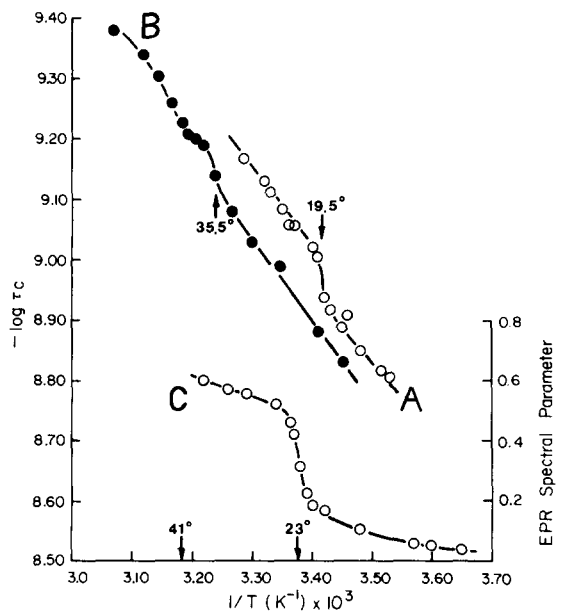

Fig. 2. Arrhenius treatment of headgroup spin label correlation time (τ_c) data for globoside at 3 mol% in unsonicated bilayers of (A) dimyristoyl phosphatidylcholine and (B) dipalmitoyl phosphatidylcholine. The phase transition temperatures of these phospholipids (23 and 41°C, respectively) are indicated on the abscissa axis; while curve (C) referred to the R-hand ordinate axis is a TEMPO spectral parameter plot [23] illustrating the fact that 3 mol% globoside does not appreciably shift the host matrix transition temperature. Samples were run in Hepes-buffered isotonic saline pH 7.4.

rather than globoside [7]. A second possible explanation is that glycosphingolipids create localized defects in the otherwise crystalline phospholipid matrix because of a failure to 'fit' optimally and/or because of a tendency to be segregated (thus increased in local concentration). There have been reports from other groups in addition to our own suggesting the possibility of ganglioside clustering in bilayers of synthetic phospholipids [10,25,26].

Another feature of the curves in Fig. 2 is that the magnitude of the effect on oligosaccharide dynamics of abrupt conversion of the host matrix from a rigid state to a very fluid state is quite small. This is reflected both in the modest size of the curve inflections and in the relative constancy of the overall curve slope. Since the data are plotted in an Arrhenius format as 'log rate' vs. $1/T$, the slopes of the curves are related to activation energy for headgroup label reorientation. The slopes of the plots Fig. 2A and B are the same above and below the region of phase transition-calculated E_{act} about 5.4 kcal/mol (the slopes are also independent of host matrix composition, being the same for dimyristoyl and dipalmitoyl phosphatidylcholine). In short, globoside headgroup motional characteristics seem only modestly correlated with phenomena affecting the hydrophobic portion of the glycolipid. This is in agreement with

our previous observations on headgroup labelled gangliosides in which we derived a value of 5.8 kcal/mol for E_{act} of label reorientation [7]. Arrhenius-type plots of this sort are in marked contrast to those typical of membrane processes such as transport [27,28] which are very sensitive to membrane fluidity and characteristically show dramatic slope changes associated with membrane melting.

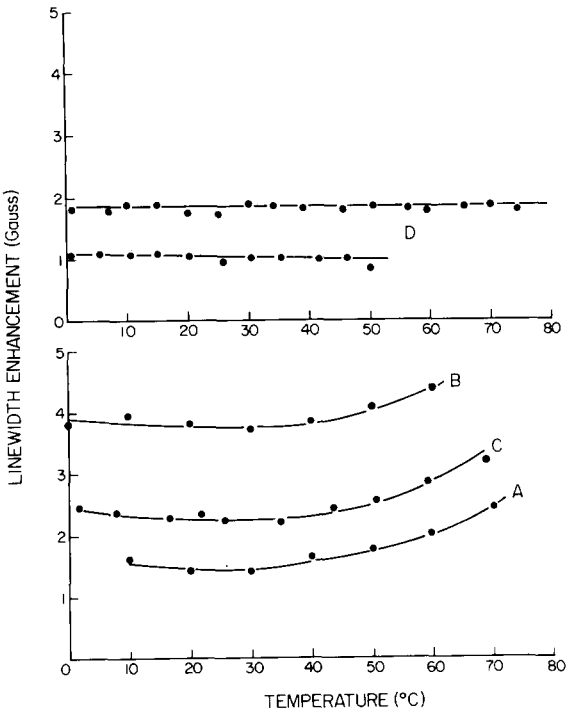

Fig. 3. Spectral line broadening induced by nitroxide-nitroxide interaction, as a function of temperature for various spin labelled lipids in bilayers of egg phosphatidylcholine. (A) 10 mol% spin labelled phosphatidylcholine - spin label one carbon from the methyl end of the β-chain fatty acid (the (1,14) label [19]). (B) same as (A) but with the spin label 12 carbons from the methyl end of the β-chain fatty acid (the (12,3) derivative). (C) 10 mol% spin labelled galactosyl ceramide - spin label at the (1,14) position of the fatty acid [29]. (D) the same experiment performed with bovine brain gangliosides labelled on random headgroup sugars [4,7] at 5 mol% (lower) and 10 mol% (upper). Line broadening was measured relative to similar samples containing only 0.5 mol% spin labelled derivative in egg phosphatidylcholine. All samples were run in Hepes-buffered saline, pH 7.4, without Ca^{2+} or Mg^{2+}.

Role of the ceramide backbone in determining headgroup behaviour

In dealing with the previous section, the question arose as to the organizational status of the ceramide backbone in a phospholipid bilayer matrix. Perhaps the most straightforward way to approach this problem is to look for differences between the behaviour of a phospholipid and a glycosphingolipid in the same matrix. For this purpose we chose derivatives with spin labels covalently attached to the fatty acid chains of phosphatidylcholine and galactosyl ceramide, respectively (the latter is the only glycolipid so labelled to date and its route of synthesis is not necessarily applicable to more complex glycolipids [29]). The spectral parameter examined was line broadening induced by nitroxide-nitroxide interactions. This phenomenon has been analysed in detail for spin labelled phospholipids in phospholipid bilayers [30]. Devaux et al. [30] demonstrated, using a phospholipid spin labelled near the methyl end of the fatty acid chain ((1,14) fatty acid) that at low temperature dipolar interactions predominate as the source of nitroxide-nitroxide line broadening. The same authors showed that, with increasing temperature, dipolar interactions are averaged out (which tends to narrow the spectral lines), but that spin exchange effects increase with temperature and lead to an overall progressive line broadening. Fig. 3 illustrates our results for the same experiment-which are in agreement with those mentioned above. Note that a very similar curve was obtained with a phospholipid spin labelled near the headgroup region ((12, 3) fatty acid) - this is consistent with the interpretation that the curve shape is governed by lipid lateral diffusion [30]. Note also that a strikingly similar curve was obtained using spin labelled galactosyl ceramide in phospholipid bilayers in spite of the fact that the phenomena determining curve shape should be highly sensitive to molecular arrangement and collision frequency. The message seems to be that, at least in fluid lipid bilayers, the ceramide backbone itself behaves in a fashion comparable to that of a phospholipid and is unlikely to be the source of any peculiarities of headgroup behaviour. Fig. 3D shows, however, that when similar experiments were performed with headgroup spin labelled gangliosides the inter-molecular line broadening remained constant over a wide temperature range.

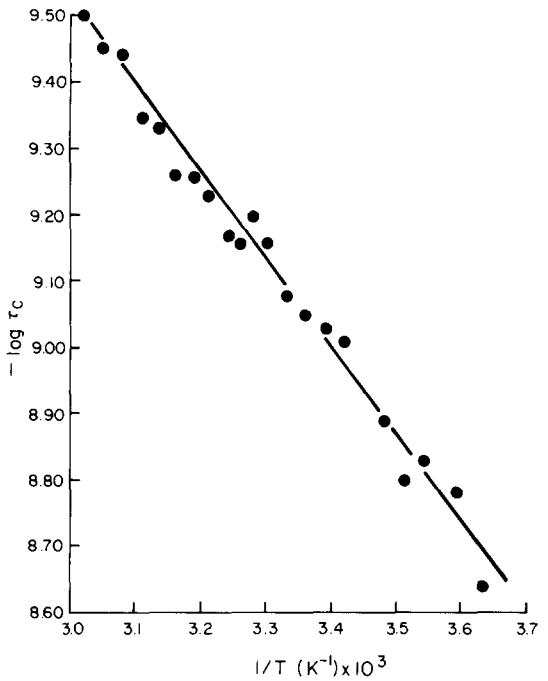

Fig. 4. Arrhenius treatment of correlation time (τ_c) data obtained with asialoglycophorin in bilayers of dimyristoyl phosphatidylcholine. Lipid: protein mol ratio 170: 1. Hepes-buffered isotonic saline, pH 7.3/2 mM $CaCl_2/MgCl_2$.

Influence of N-acetylneuraminic acid on oligosaccharide headgroup dynamics

As stated at the outset, a potentially significant difference between globosides and gangliosides is the absence of *N*-acetylneuraminic acid residues in the former. However, it is clear from the experiments so far described that we have been unable to demonstrate any significant difference in mobility or environment between the headgroups of these two families of glycosphingolipids. It seems that this observation may be a general one. We have previously reported that the correlation times for spin labelled sugar residues on the sialic acid-rich membrane protein, glycophorin, remain essentially the same following desialylation with HCl [12]. Also, the activation energy of reorientation of the spin label, obtained from an Arrhenius plot (Fig. 4) for desialylated glycophorin is 5.9 kcal/mol, which is similar to the values obtained for glycolipids in Fig. 2 and also within experimental error of our previously reported value of 6.1 kcal/mol for the native glycoprotein labelled on *N*-acetylneuraminic acid residues [12].

Further evidence that *N*-acetylneuraminic acid residues have little influence on headgroup dynamics was provided by a study of spin labelled intestinal mucins. These are glycoproteins of molecular weight $1-2 \cdot 10^6$ which are composed of some 70% (by weight) or more oligosaccharide, linked to a peptide core through *O*-glycosidic bonds (reviewed in Ref. 31). Because of their high carbohydrate content, intestinal mucins gel at concentrations above about 10 mg/ml. Their biological function is thought to involve lubrication and protection of the epithelial surface of the gut.

Human intestinal mucin was spin labelled on *N*-acetylneuraminic acid, or Gal and GalNAc res-

TABLE II

Typical spectral data (spin label correlation time, τ_c, and hyperfine splitting, a_N) for aqueous solutions of various glycoproteins labelled on either *N*-acetylneuraminic acid (NANA) residues or Gal/GalNAc data for human intestinal mucins are given at two different concentrations - non-viscous solution and gel - to illustrate the relative lack of effect of oligosaccharide crowding on sugar freedom of motion. Values of a_N for the (free) nitroxide spin label used in labelling glycoproteins are 16.8 G in buffer and 14.5 G in hexane.

Sample	Glycoprotein concentration (mg/ml)	Sugar spin labelled	τ_c ($\pm 0.5 \cdot 10^{-10}$ S)	a_N (± 0.2 G)
Glycophorin	0.6	NANA	9.6	16.8
Asialoglycophorin	0.6	Gal	9.0	16.8
Human intestinal mucin	2.5	NANA	15.1	16.8
Human intestinal mucin	14.0	NANA	15.8	16.8
Human intestinal mucin	2.5	Gal/GalNAc	10.9	16.8
Human intestinal mucin	14.0	Gal/GalNAc	12.5	16.8

TABLE III

Typical spectral data for headgroup-labelled globoside and bovine brain ganglioside in lipid bilayers with and without a dense surface covering of tightly bound 'peripheral protein' or oligosaccharide. Adsorbed layers of protein (serum albumin) and oligosaccharide (Dextran T 90) were stable indefinitely due to prior covalent attachment of fatty acid chains (see text). All samples were prepared in Hepes-buffered saline pH 7.4/2 mM $MgCl_2/CaCl_2$. Values of a_N for the (free) nitroxide spin label used in labelling glycolipids are 16.8 G in buffer and 14.5 G in hexane. Coated bilayers were prepared by hydration in the presence of 2 mg/ml of the fatty acid-conjugated albumin or Dextran - conditions which give good surface coverage as determined with radiolabelled derivatives.

Conditions	Globoside		Ganglioside	
	τ_c ($\pm 0.5 \cdot 10^{-10}$ S)	a_N (± 0.1 G)	τ_c ($\pm 0.5 \cdot 10^{-10}$ S)	a_N (± 0.1 G)
5 mol% in egg phosphatidylcholine	12.7	16.8	14.6	16.8
5 mol% in egg phosphatidylcholine + surface layer of protein	12.7	16.8	14.6	16.8
5 mol% in egg phosphatidylcholine + surface layer of oligosaccharide	12.7	16.8	14.8	16.8

idues and studied in aqueous solution (Table II). The correlation times and hyperfine splittings are consistent with the data seen for globoside and gangliosides in Table I. Note that the physical state (gel or sol) - hence oligosaccharide crowding - has little effect on head group parameters. Similar results have been obtained with bovine submaxillary mucin by Aplin et al. [16].

Non-specific headgroup interaction with peripheral protein and oligosaccharide

Glycolipid headgroups occupy a layer extending up to 25 Å from the bilayer/water interface. In cell membranes this is the innermost glycocalyx, and is densely populated by membrane proteins, oligosaccharide chains and macromolecules adsorbed from the surrounding medium. It seems a reasonable likelihood that the presence of such material may have important consequences for headgroup behaviour - if only as a result of direct collisional interaction. It has been suggested for instance that such phenomena may be involved in determining lateral diffusion of receptors [32]. In an attempt to investigate these considerations in a controlled fashion we have prepared derivatives of Dextran and of serum albumin that have covalently attached fatty acid chains [14,15]. The resultant macromolecules, when incubated with lipid bilayer membranes, adsorb spontaneously and are not removed by washing even over a period of many hours (unpublished data). The question we have asked here is whether interaction with such a strongly attached surface layer of 'peripheral protein' or carbohydrate might influence the head groups of glycolipids sharing the same bilayer membrane - and whether charged (ganglioside) headgroups might be more or less affected than neutral (globoside) headgroups. A partial answer to this question may be found in Table III: the values of spin labelled headgroup correlation time and hyperfine splitting constants are remarkably unaffected by the presence of a complete surface coverage of tightly-bound protein or polysaccharide. It is important to keep in mind though, that the spin label techniques employed here monitor the overall spin label motion which is undoubtedly a combination of the fast rotation of a terminal sugar about its linkage, superimposed upon slower waggings, gyrations, and diffusional motions of entire chains and headgroups (this point has also been made by others [33,34]). Hence, in the face of continued rapid segmental motions our experiments may fail to record alterations in more gross (yet very interesting) headgroup behaviour.

Acknowledgements

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