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A MODEL FOR GANGLIOSIDE BEHAVIOUR IN CELL MEMBRANES

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Summary

Gangliosides from beef brain have been spin-labeled using two different attaching groups and employed to investigate the physical nature of ganglioside behaviour in membranes. Results obtained using EPR spectroscopy indicate that, in phosphatidylcholine bilayers at physiological pH, ganglioside oligosaccharide chains are quite mobile and show a measurable tendency towards cooperative interaction amongst themselves. We suggest that the source of this interaction is the formation of H-bonds between sugar residues in adjacent ganglioside molecules. We present evidence that physiological (extracellular fluid) levels of Ca^{2+} and Mg^{2+} lead to cross-linking and condensing of ganglioside headgroups by complexing sialic acid carboxyl residues. Ganglioside headgroup interactions are not very sensitive to changes in the buffer ionic strength, suggesting that ionic interactions are of minor importance. We have found no measurable tendency for headgroup carbohydrate to penetrate hydrophobic regions of lipid bilayers. EPR spectroscopy was also used to follow the interaction of spin-labeled gangliosides with the glycoprotein, glycophorin, and with intact BHK cells.

We suggest that these carbohydrate-based interactions should contribute significantly to the properties of the eucaryotic cell glycocalyx. We predict that laterally mobile carbohydrate-bearing components of cell surfaces will show a tendency to cluster about complex glycoprotein arrays, especially if the species involved bear accessible carboxylic acid functions.

Introduction

Gangliosides are an important lipid component of the carbohydrate-rich layer (the glycocalyx [1]) known to surround mammalian cells. Relatively little is known about the structure and dynamics of this layer, but use of spin-label spectroscopic probes can begin to answer some of the questions involved.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

For many purposes the ideal spectroscopic probe is one which is localized in a known region and sensitive to changes only in that region. One would like such a probe to be highly sensitive to various aspects of the environment, mobility, and interactions of a given molecule, in systems as closely approximating the intact cell as possible. Spin labels have repeatedly been shown to possess these characteristics and have been used extensively in biomembrane research. Covalent attachment of spin labels to glycolipids [2—5] permits one to monitor their behaviour and surroundings in detail. The approach of rebuilding isolated membrane components, including spin-labeled gangliosides, into lipid bilayers of increasing complexity allows one to focus on individual factors involved in membrane-based events.

Materials and Methods

Beef brain gangliosides were isolated by a modification of the method of Kanfer [6] in which the crude gangliosides obtained from the initial Folch extraction were purified by silicic acid column chromatography (Bio Rad 200—325 mesh) eluting with methanol/chloroform (20—70%). Thin layer chromatography on silica gel G (Stahl) showed the pure gangliosides to be a mixture of mono-, di- and tri-sialo species.

Egg phosphatidylcholine was purchased from Sigma (Type III-E) and further purified by column chromatography on silicic acid. Phosphatidylserine was obtained from Serdary Res., London, Canada. All phospholipids were pure, as judged by thin layer chromatography on silica gel G (Stahl).

Glycophorin was isolated from freeze-dried human erythrocyte ghosts by the method of Marchesi and Andrews [7]. Ca^{2+} and Mg^{2+} were added as their chlorides, and EDTA as the disodium salt. HEPES was obtained from Sigma.

Lipid mixtures were made by dissolving appropriate amounts of each in chloroform/methanol and pumping extensively under vacuum to remove traces of solvent. Dried lipid mixtures were suspended in buffer by vortexing. EPR spectra were run on a Varian E12 spectrometer at room temperature (23°C), unless otherwise stated.

Synthesis of spin-labeled gangliosides

The phosphate-linked spin-labeled ganglioside was synthesized as described previously [5]. The preparation of this ganglioside spin label used in the experiments described below contained 0.92 labels per ganglioside, assuming an average ganglioside molecular weight of 1800.

The carboxyl-linked spin-labeled ganglioside was synthesized as follows: 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (Aldrich) was converted to the 3-carboxy derivative by the method of Rosantzev [8]. 46 mg of beef brain gangliosides and 19 mg of 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl were mixed in 0.5 ml of pyridine (CaH_2 dried) in a small tube and evaporated to dryness under vacuum. This was repeated twice more to ensure removal of traces of water. 62 mg of triisopropylbenzenesulfonyl chloride (Aldrich) in 1.0 ml of dry pyridine was added to the tube and the mixture was stirred magnetically in a sealed flask containing $CaCl_2$ for 48 h at room temperature. The reaction was terminated by addition of 2 ml of distilled water and the mixture

was dialyzed against 2 changes of distilled water prior to being evaporated to dryness. The ganglioside fraction was isolated by chromatography on a small silicic acid column, eluting with methanol/chloroform (20—70%). Column fractions were monitored by thin layer chromatography on silica gel G (Stahl) and checked by EPR spectroscopy. The ganglioside fractions were pooled, dried down and dialyzed against distilled water for 24 h at 4°C. The purified ganglioside spin label was then evaporated to dryness, redissolved in chloroform/methanol, and stored at −20°C. Yield was about 45%, based on ganglioside starting material. The extent of spin labeling of the ganglioside preparation was 0.63 per molecule of ganglioside, as estimated by double integration and comparison with a standard solution of tempocholine chloride.

Flip-flop of spin-labeled ganglioside

The flip-flop rate of phosphate-linked spin-labeled ganglioside was determined by the basic approach of Kornberg and McConnell [9]. Single bilayer egg phosphatidylcholine vesicles (250 Å diameter) containing 2 mol% phosphate-linked ganglioside spin label were prepared by sonication in 50 mM phosphate buffer, pH 7.0, at a concentration of 40 μmol lipid/ml. Approximately 8 2-min bursts of sonication with a microtip probe sonicator were required (Heat Systems Ultrasonics Model W140) with ice-bath cooling. The resulting clear suspension was centrifuged at 18000 × *g* for 20 min and the (small) pellet of titanium was discarded.

In order to determine the time necessary for complete reduction of outward-facing label, the following experiment was performed. A 50 μl aliquot of the sonicated lipid suspension was cooled to 0°C prior to addition of 10 μl of cold 90 mM sodium ascorbate, pH 7.0. The sample was immediately transferred to a pre-cooled EPR sample tube and its spectrum monitored continuously at 0°C. The intensity of the midfield peak relative to the initial intensity at time 0 (h_t/h) was measured as a function of time, and after 4 min essentially all outward-facing label was reduced.

To determine the rate of flip-flop, 1.1 ml of the sonicated lipid suspension was cooled to 0°C and incubated with 50 μl of 90 mM sodium ascorbate, pH 7.0, for 10 min. Ascorbate does not penetrate lipid bilayer vesicles at 0°C. After this ascorbate treatment (to reduce outward-facing spin labels) the sample was run down a calibrated column of Sephadex G-25 at 0°C to remove excess ascorbate. This sample was then incubated at 23°C to allow ganglioside flip-flop to occur. At various time intervals (0—5 h) 100-μl aliquots were removed, chilled to 0°C and treated with 5 μl of 90 mM sodium ascorbate for 10 min to reduce any label which had flip-flopped to the outer monolayer. The EPR spectrum of each aliquot was recorded at 0°C following this final treatment, and the peak height ratio h_t/h was measured as a function of time.

Incorporation of spin-labeled gangliosides into cultured cells

Spin-labeled gangliosides (both phosphate- and carboxyl-linked) were incorporated into cultured BHK-21 cells by two different methods: label was added to the cells either simply dissolved in buffer (where it exists as micelles) or as an integral component of single bilayer vesicles of egg phosphatidylcholine. BHK-21 cells grown in spinner culture were pelleted from the medium and

washed twice with Ca^{2+}, Mg^{2+}-free phosphate buffered saline. To 100 μl of packed cells was added either (i) 1 ml of Ca^{2+}, Mg^{2+}-free phosphate buffered saline containing 0.26—0.50 μmol of ganglioside spin label, or (ii) 1 ml of a sonicated suspension in Ca^{2+}, Mg^{2+}-free phosphate buffered saline of 40 mol% ganglioside spin label in egg phosphatidylcholine (total label 0.26—0.50 μmol). Sonicated suspensions were prepared as described in flip-flop experiments. The cell suspensions were incubated at 37°C for 2 h with periodic shaking. The sample incubated with dissolved gangliosides was washed twice with Ca^{2+}, Mg^{2+}-free phosphate buffered saline. The sample incubated with lipid vesicles was first washed with 10% (w/v) sucrose/10 mM phosphate, pH 7.0, at 37°C (to remove firmly bound lipid) and then with Ca^{2+}, Mg^{2+}-free phosphate buffered saline. The washed cell pellets were transferred to 50-μl sample tubes and EPR spectra were recorded immediately.

Results and Discussion

Spin label spectra and ganglioside headgroup mobility

The glycolipids employed in this work, whether labeled or unlabeled, represented the total purified fraction of beef brain gangliosides. In order to covalently attach nitroxide radicals (spin labels) to the headgroup region, use was made of the fact that each carbohydrate residue possesses one primary hydroxyl group. Primary alcohols were esterified to nitroxide-containing rings via an attached phosphate or carboxyl function (Fig. 1). In general, we have controlled reagent ratios so as to introduce (presumably randomly) an average of one or fewer spin labels per ganglioside. This low level of labeling should mini-

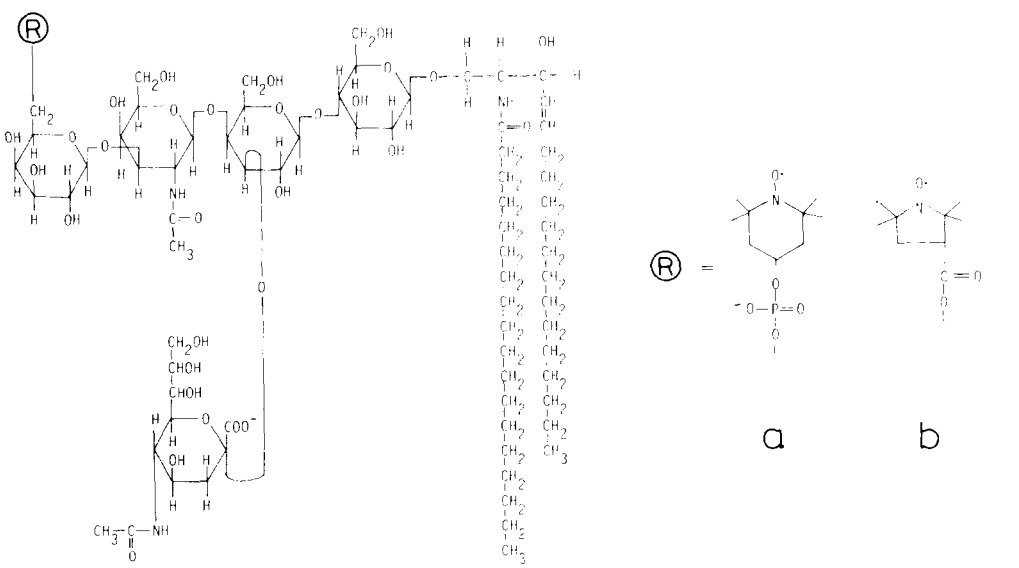

Fig. 1. Structure of a representative spin-labeled ganglioside. The attached group for the phosphate-linked label is shown in a, the carboxyl-linked label in b. The labeling procedure has a specificity for primary alcohol groups, and is assumed to have no particular preference for any sugar residue. On the average there is one or fewer spin labels per ganglioside molecule, presumably distributed randomly among the various sugars.

mize any spin label-induced perturbation and also minimize intramolecular spin exchange broadening of the EPR signal.

Several important points emerge from the spectra of the labeled gangliosides incorporated at low concentrations into lipid bilayer structures (Fig. 2). Firstly, the nitroxide moieties show very little evidence of the immobilization seen when similar nitroxide rings are bound at lipid bilayer surfaces [10] or attached to proteins [11]. We have interpreted this to indicate that the oligosaccharide portions of gangliosides are inherently unrestricted in their motion. However, the actual rate of spin-label motion (the correlation time, τ_c) can be estimated from such spectra [12] and this has been done [4,5]. Such calculations begin to break down as far as precise numbers are concerned if the motion is much slower than that associated with Fig. 2 (upper spectrum τ_c approx. $1.3 \cdot 10^{-9}$ s). But our interest has only been in approximate rates of motion and changes in mobility as a function of various external factors. Note that the carboxyl-linked label has several advantages over the phosphate-linked analogue (Fig. 1) in that there are only 2 bonds about which free rotation can occur, separating the spin label from the sugar ring (rotation about the C-0 bond should be restricted), and the attaching chain is uncharged. Reduced rotational freedom is evident in the EPR spectrum of the carboxyl-linked label (Fig. 2). The second point of interest in the spectra shown in Fig. 2 is the separation between the spectral lines. This splitting is characteristic of the polarity of the nitroxide environment, varying over some 2 G depending on whether the nitroxide is exposed to the aqueous surface of a bilayer or the hydrophobic interior [13]. The splittings observed: 16.95 G for the phosphate-linked label (compared to 17.00 G for tempophosphate in water) and 16.12 G for the carboxyl-linked label (compared to 16.15 G for the free label in water) are consistent with an aqueous environment. This would argue against the carbohydrate residues (with attached spin labels) spending appreciable amounts of time in hydrophobic environments such as the membrane interior.

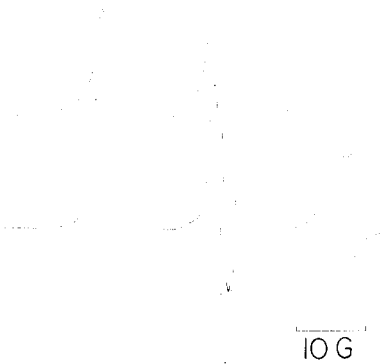

Fig. 2. EPR spectra of headgroup spin-labeled gangliosides at a concentration of 1.5 mol% in bilayers of egg phosphatidylcholine. The buffer used to hydrate the samples was 10 mM phosphate, pH 7.0. The upper spectrum is that of the carboxyl-linked label (τ_c approx. $1.3 \cdot 10^{-9}$ s), while the lower spectrum is that of the phosphate-linked label (τ_c approx. $3.5 \cdot 10^{-10}$ s).

It has been shown previously that purified glycolipids may be assembled into phospholipid bilayer structures in a fashion which closely mimics their orientation and binding function in real cells, (e.g., refs. 3, 14—16). The attraction of working with lipid bilayers into which one can reassemble purified membrane components is the potential ability to reconstruct unambiguously the complex interactions found in biological membranes. The experiments described above leave one with a picture of ganglioside headgroups protruding from the bilayer surface and being unrestricted. This conclusion is drawn from studies of fluid, neutral lipid bilayers (egg phosphatidylcholine at 23°C) in the absence of salts and in the virtual absence of glycocalyx (only some 1% ganglioside). Much of the remainder of this paper deals with the successive imposition of other factors which are of undoubted importance in real cell membranes.

Flip-flop of gangliosides

We have used the method of Kornberg and McConnell [9] to measure flip-flop rates for the phosphate-linked label in fluid lipid vesicles (egg phosphatidylcholine at 23°C). Briefly, ascorbate was added (on ice) to sonicated vesicles containing 2 mol% ganglioside spin label in order to reduce all outward-directed nitroxides. Excess ascorbate was then removed at 0°C and the vesicles were incubated at 23°C for various times prior to being chilled to 0°C for a second ascorbate treatment. For periods of up to 5 h there was no further reduction of label upon adding ascorbate for the second time, indicating that unreduced (inner surface) labels did not exchange with outer surface labels over this time course (Fig. 3). The amount of label which remained inaccessible to ascorbate (28%) is consistent with that expected from theoretical considerations [9]. Hence, as might be predicted from thermodynamic considerations, we have found no evidence of ganglioside flip-flop in fluid lipid bilayers.

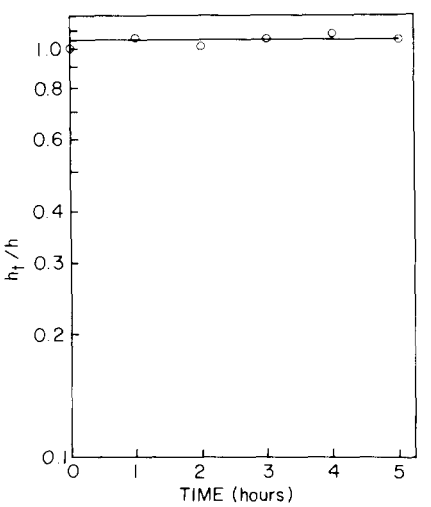

Fig. 3. Fraction of spin-labeled ganglioside which remains protected at the inner surface of sealed bilayer vesicles following an initial treatment with ascorbate at 0°C, removal of excess ascorbate, and incubation at 23°C for the time indicated. h_t/h is the ratio of the intensity of the midfield peak at time t to the intensity at time 0. An appreciable flip-flop rate would lead to a steady drop in this ratio. The lipid mixture used was egg phosphatidylcholine containing 2 mol% phosphate-linked ganglioside spin label.

Ganglioside headgroup interactions and the effect of salt

The concentration of carbohydrate (attached to lipids and proteins) at cell surfaces is considerably higher than that found at the surface of phospholipid bilayers containing 1% ganglioside. It should be noted too that our lipid bilayer systems are symmetric, whereas in real cells all the carbohydrate-bearing material is concentrated at the outer surface. In an attempt to mimic this concentration phenomenon we have examined the effect of increasing amounts of unlabeled ganglioside on a small amount of spin-labeled ganglioside in lipid bilayers. If some spectral parameter related to oligosaccharide headgroup mobility is plotted as a function of increasing ganglioside concentration in the bilayer, a sigmoidal curve is obtained (Fig. 4). Very similar results are obtained with both types of labeled gangliosides [4]. We have previously reported that the physical state of the bilayer (i.e., fluid or rigid) has relatively little effect on ganglioside headgroup mobility [4,5]. We interpret the curves in Fig. 4 as showing a cooperative ganglioside headgroup interaction leading to decreased mobility. Note, however, that even at high surface carbohydrate concentration the headgroups cannot be considered highly immobilized. Comparison of the curve measured in the presence of isotonic saline (Fig. 4, curve B) to that measured in 10 mM phosphate (Fig. 4, curve A) suggests that ionic interactions are of minor importance in this headgroup interaction. The fact that curve B does not level off as readily as that measured in dilute buffer may reflect a reduction in sialic acid negative charge repulsion at higher ionic strength.

As noted earlier, the lipid bilayers employed in these experiments are symmetric. If all the carbohydrate were on one side, as in real membranes, the effects seen should occur at half the concentration of ganglioside used here. Even so, it is obvious that the effects shown in Fig. 4 occur at ganglioside concentrations higher than those (up to some 6%) found in mammalian plasma

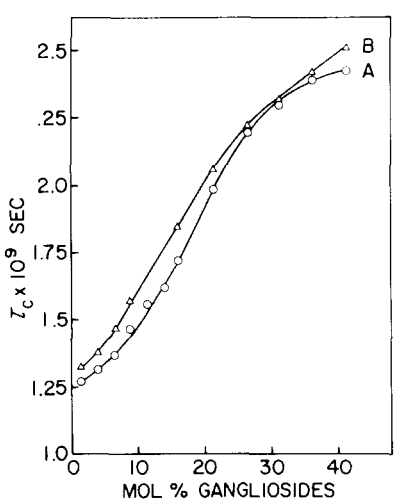

Fig. 4. Effect of increasing amounts of total ganglioside on the oligosaccharide headgroup mobility of spin-labeled gangliosides in fluid lipid vesicles at 23°C. Egg phosphatidylcholine vesicles contained 1.5 mol% carboxyl-linked ganglioside spin label, and various amounts of unlabeled ganglioside. Headgroup mobility is inversely related to the correlation time τ_c. Lipid vesicles were suspended in 10 mM phosphate buffer, pH 7.0 (curve A) or Ca^{2+}, Mg^{2+}-free phosphate buffered saline (curve B).

membranes. However, these results should provide some insight into the phenomena to be expected when a wide range of different oligosaccharide chains (whether on glycolipids or glycoproteins) are confined to a membrane surface, as is the case for cell plasma membranes.

Interaction of gangliosides in solution with divalent cations and glycophorin

The divalent cation, Ca^{2+}, is known to be important in the maintenance of mammalian cell membrane integrity and function (e.g., ref. 17). It is found at concentrations of 1—2 mM in extracellular fluid and is known to complex very strongly with carboxyl residues [17]. Addition of Ca^{2+} to (spin-labeled) gangliosides free in solution leads to broadening of the EPR signal (see Table I). In all of the experiments described here the gangliosides are above their critical micelle concentration of 0.2 mg/ml [18]. The source of the spectral broadening seems to be ganglioside headgroup-crosslinking by divalent cations leading to decreased headgroup mobility and an increased incidence of collision between nitroxide radicals (spin exchange broadening [19]). The relative contributions of these two broadening mechanisms (immobilization and spin exchange) may be estimated by substituting unlabeled ganglioside for the bulk of the labeled ganglioside in the same experiments. The result (Table I) is that the spin exchange effect is greatly reduced. The data indicate that the spectral effect seen is largely (up to 80%) spin exchange broadening rather than immobilization. It seems likely that the observed divalent cation crosslinking of ganglioside headgroup sialic acid residues might also have a secondary condensing effect by bringing the carbohydrate residues into closer contact and increasing the chance for favourable interactions such as H-bonding.

Ganglioside-glycoprotein interactions are evident from experiments in which

TABLE I

EFFECT OF Ca^{2+} ON GANGLIOSIDES AND GLYCOPHORIN IN BUFFER

The carboxyl-linked ganglioside spin label was used in all samples. Total ganglioside concentration was 2 mg/ml, of which all was labeled for the first group of samples (A), but only $\frac{1}{4}$ was labeled for the low label samples (B). Concentrations of other reagents were as follows: glycophorin, 20 mg/ml; EDTA, 5 mM; Ca^{2+}, 100 mM. Buffer used was 5 mM HEPES, pH 7.0.

Sample	Lowfield linewidth (G)	Linewidth difference Ca^{2+}—EDTA (G)
A. Gangliosides + EDTA	3.05 *	0.42
Gangliosides + Ca^{2+}	3.47	
Gangliosides + glycophorin + EDTA	2.75	1.175
Gangliosides + glycophorin + Ca^{2+}	3.925	
B. Low label gangliosides + EDTA	3.15	0.17
Low label gangliosides + Ca^{2+}	3.32	
Low label gangliosides + glycophorin + EDTA	2.94	0.25
Low label gangliosides + glycophorin + Ca^{2+}	3.19	

* Linewidths between the two groups of samples A and B should not be rigorously compared because of a difference in machine settings. However, linewidth difference values can be compared between the two groups.

both are dissolved in buffer. In the experiments described above, spectra of buffer solutions of spin-labeled gangliosides in the presence or absence of the divalent cation, Ca^{2+}, were examined. These experiments have been repeated in buffers which also contain glycophorin (the 31000 M_r integral membrane glycoprotein from human erythrocytes [20]). Glycophorin is very water-soluble once isolated, presumably by virtue of its high carbohydrate content (some 60% by weight). As Table I shows, an approximately equimolar concentration of glycophorin added to spin-labeled gangliosides in solution has a noticeable narrowing effect on the spectral lines. This would be expected if glycophorin forms mixed micelles with the gangliosides and effectively dilutes out the nitroxide-labeled sugars. Addition of Ca^{2+} to ganglioside/glycophorin mixtures leads to considerably more dramatic spectral changes than when added to gangliosides alone (see Fig. 5). These results show that gangliosides and glycophorin free in solution interact in some way via their carbohydrate and/or hydrophobic portions, and that this interaction increases the magnitude of the divalent cation effect on ganglioside headgroups. Since glycophorin is rich in sialic acid, it would be expected to take part in Ca^{2+}-crosslinking of carboxyl residues.

It is surprising that a relatively large fraction of the spectral effect caused by Ca^{2+} addition to solutions of glycophorin and spin-labeled ganglioside is spin exchange broadening. Also, the magnitude of the effect is even larger than in the absence of the glycoprotein, in spite of the fact that the large amount of carbohydrate contributed by the latter is all unlabeled. This is consistent with the idea that the large, dense masses of carbohydrate on glycophorin serve as a focal point which increases the extent of ganglioside packing in the presence of Ca^{2+}.

Ganglioside-divalent cation effects in lipid bilayers

In order to extrapolate the findings reported in the previous section to cell membranes we have re-examined the same phenomena with components incorporated into lipid bilayers. Both Ca^{2+} and Mg^{2+} have dramatic effects on the EPR spectra of headgroup-labeled gangliosides in phosphatidylcholine bilayers

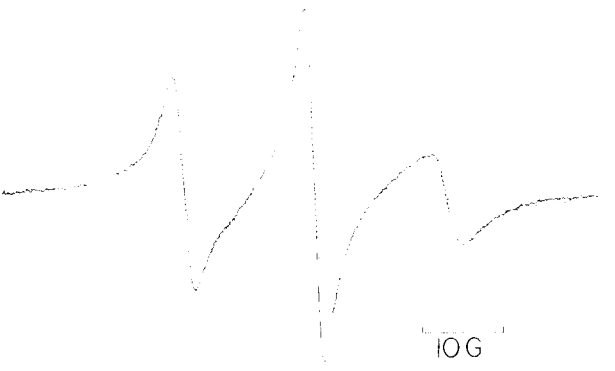

Fig. 5. EPR spectra of carboxyl-linked ganglioside spin label and glycophorin in buffer containing either 5 mM EDTA (solid line), or 100 mM Ca^{2+} (dotted line). The concentration of spin-labeled gangliosides and glycophorin was 2 mg/ml and 20 mg/ml respectively. Buffer used was 5 mM HEPES, pH 7.0.

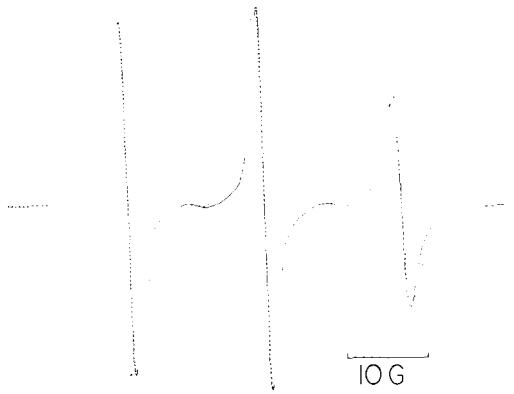

Fig. 6. EPR spectra of phosphate-linked ganglioside spin label at a concentration of 5 mol% in vesicles of egg phosphatidylcholine. The suspending buffer was 5 mM HEPES, pH 7.0, containing either 5 mM EDTA (solid line) or 100 mM Ca^{2+} (dotted line).

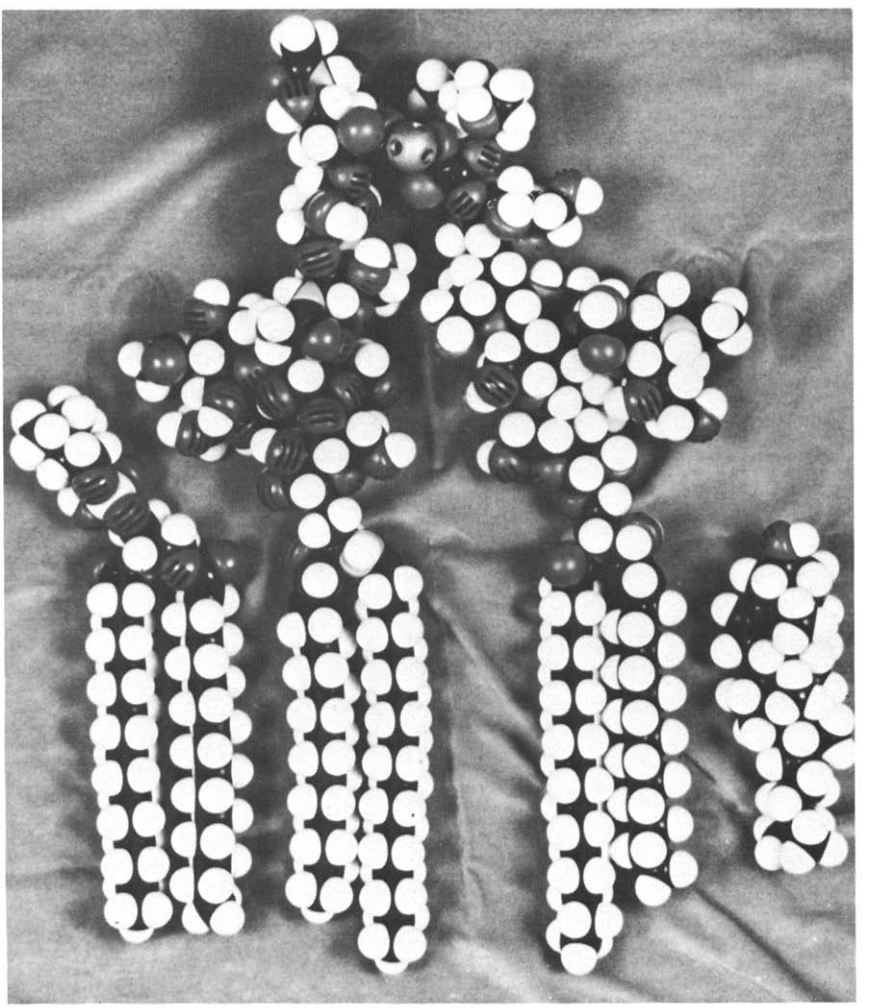

Fig. 7. Space-filling models (P.C.K.) of two ganglioside molecules crosslinked by a single divalent cation (Ca^{2+} or Mg^{2+}) through sialic acid residues. The gangliosides shown are both GD_{1a} but present different faces to the viewer. Models of phosphatidylcholine and cholesterol are shown to the left and right respectively for comparison. Formation of intramolecular divalent cation crosslinks appears sterically and energetically unfavourable.

TABLE II

EFFECT OF Ca^{2+} ON GANGLIOSIDES IN PHOSPHOLIPID BILAYERS

Samples contained 2.6 μmol of 5 mol% labeled ganglioside in egg phosphatidylcholine (egg PC) or phosphatidylserine (PS). Low label samples consisted of 0.5 mol% labeled and 4.5 mol% unlabeled ganglioside. Samples were vortexed in 50 μl of either 5 mM EDTA, 100 mM Ca^{2+} (egg phosphatidylcholine) or 200 mM Ca^{2+} (phosphatidylserine samples), all in 5 mM HEPES buffer, pH 7.0. The low Ca^{2+} sample was suspended in 2.5 ml of 2 mM Ca^{2+} (this contains the same total amount of Ca^{2+} as 50 μl of 100 mM Ca^{2+}) and incubated for 2 h at room temperature. The lipid was pelleted at 18 000 × g for 20 min and resuspended in 50 μl of supernatant.

Sample	Lowfield linewidth (G)	Linewidth difference Ca^{2+}—EDTA (G)
Carboxyl-linked label		
Gangliosides-egg PC + EDTA	2.22	0.215
Gangliosides-egg PC + Ca^{2+}	2.435	
Low label gangliosides-egg PC + EDTA	1.99	0.04
Low label gangliosides-egg PC + Ca^{2+}	2.03	
Gangliosides-egg PC + large vol. low Ca^{2+}	2.33	0.11
Phosphate-linked label		
Gangliosides-egg PC + EDTA	1.82	0.295
Gangliosides-egg PC + Ca^{2+}	2.115	
Low label gangliosides-egg PC + EDTA	1.80	0.09
Low label gangliosides-egg PC + Ca^{2+}	1.89	
Gangliosides-PS + EDTA	1.855	0.185
Gangliosides-PS + Ca^{2+}	2.04	
Low label gangliosides-PS + EDTA	1.84	0.185
Low label gangliosides-PS + Ca^{2+}	2.025	

(Figs. 6 and 7, Tables II and III). This is true for both phosphate- and carboxyl-linked labels. As was observed for gangliosides free in solution, the spectral effect is predominantly spin exchange broadening indicating ganglioside clustering. Note that in most of our samples, for convenience, we have added small volumes of buffer containing high concentrations of cation to dried lipids. The

TABLE III

EFFECT OF Mg^{2+} ON GANGLIOSIDES IN PHOSPHOLIPID BILAYERS

Samples contained 2.6 μmol of 5 mol% labeled ganglioside in egg phosphatidylcholine (egg PC). Low label samples consisted of 0.5 mol% labeled and 4.5 mol% unlabeled ganglioside. Samples were vortexed in 50 μl of either 5 mM EDTA or 100 mM Mg^{2+}, both in 5 mM HEPES buffer, pH 7.0.

Sample	Lowfield linewidth (G)	Linewidth difference Mg^{2+}—EDTA (G)
Carboxyl-linked label		
Gangliosides-egg PC + EDTA	2.20	0.225
Gangliosides-egg PC + Mg^{2+}	2.425	
Low label gangliosides-egg PC + EDTA	1.955	0.025
Low label gangliosides-egg PC + Mg^{2+}	1.98	
Phosphate-linked label		
Gangliosides-egg PC + EDTA	1.86	0.35
Gangliosides-egg PC + Mg^{2+}	2.21	
Low label gangliosides-egg PC + EDTA	1.805	0.065
Low label gangliosides-egg PC + Mg^{2+}	1.87	

more "physiological" experiment is to add a large volume of buffer containing the concentration of Ca^{2+} or Mg^{2+} (1—2 mM) found in extracellular fluid. These two different approaches have very similar effects: for example, a large volume of 2 mM Ca^{2+} gives rise to more than 50% of the effect produced by a small volume of 100 mM Ca^{2+} containing the same total amount of Ca^{2+} (Table II).

The ability of Ca^{2+} (but not Mg^{2+}) to crosslink neighbouring phosphatidylserine molecules via its affinity for the headgroup carboxyl functions is known [21—23]. The fact that we find Mg^{2+} to be equally effective (compared to Ca^{2+}) at crosslinking ganglioside headgroups presumably indicates that steric constraints for crosslinking are less severe in this case. The effect of Ca^{2+} on gangliosides in phosphatidylserine bilayers is mainly immobilization rather than the clustering seen in phosphatidylcholine bilayers (Table II) and presumably results from carbohydrate crosslinking to the bilayer surface by Ca^{2+}. *

Preliminary experiments in which glycophorin is incorporated into bilayers containing low concentrations of ganglioside spin labels indicate that the gangliosides are partially immobilized by the glycoprotein, as they are when large amounts of unlabeled gangliosides are present.

Incorporation of spin-labeled gangliosides into mammalian cell membranes

It has been known for some time that when free gangliosides are added to the medium bathing mammalian cells a small fraction becomes in some way attached to the cell surface. If such gangliosides insert themselves into the membrane in a natural fashion (this is by no means a proven fact) a mechanism exists for adding specific spin-labeled gangliosides to the outer surface of intact viable cells.

BHK-21 cells were used for a preliminary investigation of spin-labeled ganglioside addition to the plasma membrane of intact cells. Following incubation of washed cells with ganglioside spin labels (either phosphate- or carboxyl-linked), the excess was removed by further washing. In the experiments described here the presence of 2 mM $K_3Fe(CN)_6$ had no apparent effect on the signal height of labeled cells; however, in some cases it may prove desirable to add this oxidizing agent to prevent reduction of the spin label nitroxide moiety. The basic observation made from these experiments is that spin-labeled gangliosides incorporated into cell membranes show clear spectral immobilization features, similar to those already described for labeled gangliosides interacting with other glycolipids or glycoproteins. This must reflect interaction of the ganglioside headgroups with components of the BHK-21 cell glycocalyx. Analogous results were obtained when spin-labeled gangliosides were incorporated into BHK-21 cells by fusion with phosphatidylcholine vesicles containing the label.

Implications for the eucaryotic cell surface

Given the forces described above, we would expect gangliosides at the eucaryotic cell surface to cluster around one another and around large masses of carbohydrate such as those found on certain glycoproteins (Fig. 8). Ganglioside-glycoprotein associations should be particularly strong in the case of

* See Note added in proof.

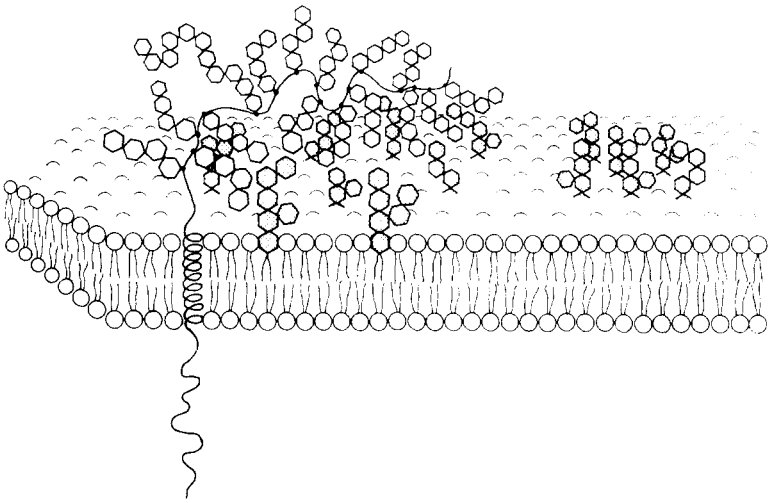

Fig. 8. Scale drawing illustrating the proposed ganglioside-ganglioside and ganglioside-glycoprotein clustering phenomena in a hypothetical fluid bilayer membrane. The single glycoprotein has been drawn to the specifications of a glycophorin monomer. Carbohydrate residues are indicated by hexagons (shaded for gangliosides). Two clusters of carbohydrate-bearing components are shown: a large mass centred about the glycophorin, and a smaller one of gangliosides alone. Note that such clusters could contain various receptors and several copies of each. They could also contain the same receptor (e.g. for influenza or Sendai virus) on both glycoproteins and glycolipids. H-bonding and divalent cation crosslinking should contribute significantly to formation of such clusters at cell surfaces.

glycoproteins bearing carboxyl residues (e.g. on sialic acid or acidic peptides), where crosslinking by divalent cations can occur. Similar considerations should contribute significantly to glycoprotein-glycoprotein interactions in cell membranes.

The glycoprotein in Fig. 8 has been chosen to fit the specifications [20] of glycophorin from human erythrocytes. We have made no allowance for glycophorin dimerization or the presence of other glycolipids or glycoproteins. Even so, one quickly arrives at a sizeable mass of intertwining oligosaccharide chains. In real cells, where the glycocalyx can be hundreds of Å thick, such individual masses (perhaps anchored by cytoskeletal constraints) could be crosslinked to neighbouring masses to form a structurally-sound coat. An important point to keep in mind however, is that the forces described here are non-covalent and could break under appropriate stress and reform when the stress is removed, much like a 3-dimensional "zipper".

Both gangliosides and glycoproteins have been implicated as receptor sites at cell surfaces, the carbohydrate portion being instrumental in determining specificity. Surface aggregations of oligosaccharide chains, such as those envisaged here, might carry a variety of receptors and several copies of each. Moreover, such structures would be highly deformable and could even be broken up by a given binding event. Hence binding kinetics can often be expected to deviate from "hard sphere" approximations and to take on a cooperative nature [24]. The same considerations suggest a simple mechanism whereby binding of one agent, especially in large amounts, could result in altered expression of plasma membrane receptors for another agent, e.g. the effect of large amounts of cholera toxin on binding of the Fc portion of immunoglobulins (see ref. 25 and references contained therein). The effect of proteolysis on protease-insensitive

receptor molecules clustered around a protease-sensitive glycoprotein could also be very dramatic.

Neuraminidases have been considered in some detail with regard to both their roles in viral membranes and their effects on cells [26]. Neuraminidase-effected cleavage of *N*-acetyl-neuraminic acid (sialic acid) from mammalian cell surfaces is known to influence cell social behaviour and recognition-related events [27]. In our model, removal of sialic acid will lead to a considerable reduction in the structural integrity of at least some regions of the glycocalyx by destroying divalent cation corsslinking. Neuraminidase-induced weakening or destruction of local areas of the host cell glycocalyx could contribute to both initial infection, by removing a barrier to the fusion event, and budding, by allowing a patch of the membrane bearing viral proteins to bleb free of the surrounding surface coat.

Note added in proof (Received November 30th, 1977)

Experiments with the carboxyl-linked label in phosphatidylserine-phosphatidylcholine bilayers indicate that Ca^{2+}-mediated ganglioside-phosphatidylserine interactions are important and can involve both clustering and immobilization.

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