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Glycosphingolipids in Membrane Architecture

Frances J. Sharom and Chris W. M. Grant

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1

As part of a program to investigate the behavior and interactions of glycolipids in biological membranes we have synthesized spin-labeled derivatives of 2 families of carbohydrate-bearing ceramides (glycosphingolipids): simple neutral glycolipids and gangliosides. Galactosyl ceramide has been synthesized with the spin label at 3 different positions on the fatty acid chain. It has been studied in bilayers of various different lipids and lipid mixtures and compared to the corresponding phospholipid spin labels. Considerable similarity has been found between the behavior of galactosyl ceramide and phosphatidylcholine. These similarities include a negligible flip-flop rate, a flexibility gradient in the acyl chains, and exclusion from phosphatidylserine domains in the face of a Ca^{2+}-induced lateral phase separation. Evidence for dramatic clustering of simple neutral glycolipids has not been found. Glycosphingolipids do seem to have the capacity to increase rigidity in fluid lipid bilayers. A general procedure has been developed for covalent attachment of a nitroxide spin label to the headgroup region of complex glycolipids such as gangliosides. Studies of beef brain gangliosides labeled in this manner and incorporated into bilayers of phosphatidylcholine indicate that the headgroup oligosaccharides are in rapid, random motion as opposed to being in any way immobilized. This headgroup mobility depends very little on the fluidity or rigidity of the bilayer. However, headgroup mobility decreases, perhaps as a result of cooperative headgroup interactions, with increasing bilayer concentration of unlabeled ganglioside.

Key words: **glycosphingolipids, membrane structure, flip-flop, spin label, glycolipids, gangliosides**

Considerable research effort is currently being directed toward fitting individual biological membrane components into a 3-dimensional model of membrane architecture. This model has to allow not only for intricate arrangements and complex associations, but also for dramatic rearrangements and association changes. We have been approaching the

Abbreviations: EPR – electron paramagnetic resonance; GC – galactosyl ceramide; PC – phosphatidylcholine

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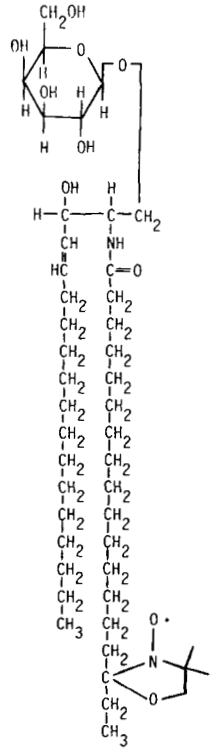
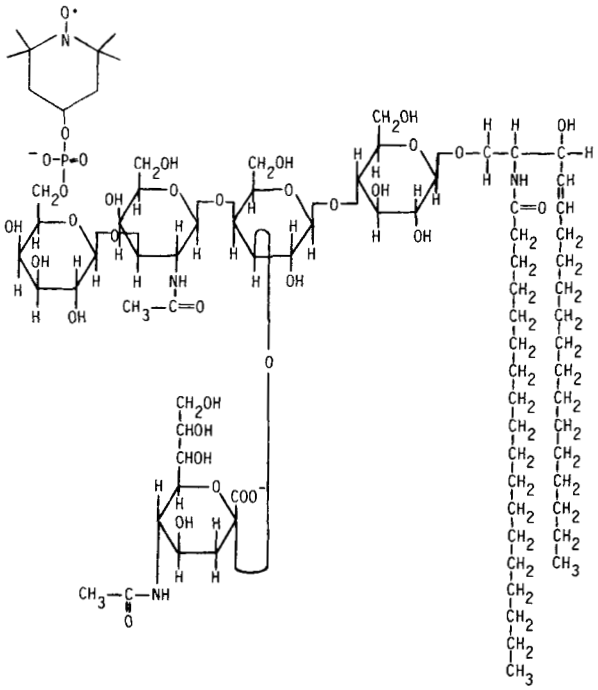
problem of glycosphingolipid behavior and interactions largely by studying spin-labeled derivatives in lipid bilayer systems. The spin label technique has proven very useful in membrane research, giving early impetus to innovative concepts such as the fluid bilayer nature of membranes (1), slow flip-flop (2), rapid lateral diffusion (3–5), and fluidity gradients (1).

The usefulness of spectroscopic probes in studies such as those outlined here is greatly increased if they are covalently attached in some clearly understood fashion to the molecule of interest. For this reason we have synthesized spin-labeled derivatives of 2 glycolipid families: simple neutral glycosphingolipids (6) and gangliosides (7) (Fig. 1). Incorporation of such labeled glycolipids into bilayers containing various mixtures of lipids is straightforward. The fate and behavior of the spin-labeled component can then be followed via its EPR[1] spectrum.

Glycosphingolipids (lipids which have carbohydrate headgroups glycosidically linked to a sphingosine backbone) are of considerable interest as outer surface components of mammalian cells. They have been implicated in a wide variety of processes related to recognition, adhesion, and growth control. An immediate question is whether they can be fitted into current membrane models in the same way as phosphatidylcholine or whether



SPIN-LABELED GALACTOSYL CERAMIDE



SPIN-LABELED GANGLIOSIDE

Fig. 1.

one may expect behavior peculiar to glycosphingolipids (i.e., as a result of the sphingosine backbone or the carbohydrate headgroup).

MATERIALS AND METHODS

For the preparation of the spin-labeled neutral glycolipid the fatty acid of natural GC was removed by base hydrolysis and replaced with 1 of 3 spin-labeled fatty acids (6). Corresponding phospholipid labels were prepared by acylation of palmitoyl lysophosphatidylcholine with the same 3 spin-labeled fatty acids (7). Beef brain gangliosides were labeled in the headgroup region by covalent attachment of the small spin label tempophosphate (8). This reaction relies on the formation of phosphate esters with primary alcohols (of which there is 1 per carbohydrate residue). The reagent ratios can be controlled and generally we have employed conditions which give 1 or fewer spin labels per ganglioside.

Antisera to natural GC were raised in rabbits as outlined by Alving et al. (9). The immunoglobulin fraction was purified by 3 successive ammonium sulfate precipitations and was then redissolved in isotonic saline at the original serum concentration. Antisera were kindly titred by Dr. Carl Alving of the Walter Reed Army Medical Center, Washington, DC.

Lipid sources and purification procedures were as described elsewhere (6, 8, 10). Lipid mixtures were made by dissolving appropriate amounts of each in $CHCl_3$ of $CHCl_3/CH_3OH$ and drying extensively in vacuo prior to rehydration.

The flip-flop rate of galactosyl ceramide in egg PC was determined by the basic approach of Kornberg and McConnell (2) but using GC derivatives spin labeled in the fatty acid chain. Flip-flop studies via ascorbate reduction of fatty acid labeled phospholipids have been reported by other workers (11). Single bilayer egg PC vesicles (~ 250° Å diameter) containing 4 mol % (12,3) spin-labeled GC were prepared by sonication in 50 mM phosphate buffer pH 7.0 at a concentration of 40 μmol/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 18,000 × g for 20 min and the (small) pellet of titanium discarded. We then cooled 1.1 ml of the suspension to 0°C and incubated with 0.1 ml of 90 mM sodium ascorbate pH 7.0 for 75 min. 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 (ascorbate does not penetrate lipid bilayer vesicles at 0°C). This sample was then incubated at 23°C to allow glycolipid flip-flop to occur. At various time intervals (0–5 hr) 100 μl aliquots were removed, chilled to 0°C, and treated with 8 μl of 90 mM ascorbate for 75 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 intensity of the low-field peak relative to the initial intensity at time 0 (h_t/h) was measured.

In order to determine the time necessary (75 min) for complete reduction of outward-facing (12, 3) spin-labeled glycolipid the following experiment was performed. Lipid vesicles containing 5 mol % GC spin label in egg PC (lipid concentration 27 μmol/ml) were made by sonication as above. A 50 μl aliquot was removed and cooled to 0°C prior to addition of 5 μl of cold 250 mM sodium ascorbate pH 7.0. The sample was immediately transferred to a precooled EPR sample tube and its spectrum followed continuously at 0°C. Monitoring the peak height ratio h_t/h as a function of time showed that after 75 min essentially all outward-facing label was reduced.

RESULTS AND DISCUSSION

The sequence of reactions used to produce (fatty acid) spin-labeled GC relies on the presence of 1 base-hydrolyzable amide ester linkage. On this basis the same reaction sequence should be capable of producing other spin-labeled glycosyl ceramides [such as glucosyl ceramide or lactosyl ceramide (12)]. A complication arises if any of the carbohydrate groups possess N-acetyl or N-glycolyl moieties which will also be subject to base hydrolysis (e.g., gangliosides, globoside). The spin label (nitroxide ring) does not interfere with the function of GC as a receptor. For instance immunoglobulins directed against natural GC dramatically agglutinate liposomes of egg PC containing 2% natural or spin-labeled GC whereas they do not affect suspensions of pure egg PC liposomes.

The technique employed to spin label gangliosides is quite general in that the requirement is only for a primary alcohol (of which there is 1 per sugar residue). Random introduction of up to 1 spin label per ganglioside headgroup adds both an extra negative charge and a new group. This should not detract too severely from extrapolation of the behavior of spin-labeled gangliosides to that of gangliosides in general since the latter are already highly negatively charged and possess a wide variety of headgroup structures.

Behavior of Neutral Glycosphingolipids

One of the first experiments we performed was to simply incorporate small amounts of spin-labeled galactosyl ceramides into fully hydrated bilayers of various phosphatidylcholines. The immediate observation was that the gross behavior of neutral glycolipids is similar to that of phospholipids (6, 10). That is, the EPR spectra (which are sensitive to mobility, orientation, spin label clustering, and environment polarity) of spin-labeled galactosyl ceramides are similar to those of the corresponding spin-labeled phosphatidylcholines. Certainly there is no dramatic clustering or immobilization of the glycolipids at concentrations of a few % in bilayers of egg PC (6). The possibility of clustering at higher concentrations of surface carbohydrate has not yet been investigated in the case of simple neutral glycolipids.

It was originally established using spin-labeled phospholipids that Ca^{2+} can induce dramatic lateral phase separations in fluid bilayers rich in phosphatidylserine (13, 14). Apparently Ca^{2+}-induced cross-linking of the headgroup carboxylic acid residues leads to formation of more rigid domains selectively enriched in phosphatidylserine which coexist with PC-enriched domains in the same bilayer (13–15). Spin labels are quite sensitive to this phenomenon because any selective pooling of the labeled component shows up clearly in spin exchange broadening of the EPR spectrum (6, 13). We have shown that GC is also subject to this Ca^{2+}-induced selective exclusion from cross-linked phosphatidylserine domains (6). In fact when Ca^{2+} is added to a suspension of liposomes containing the ternary lipid mixture, phosphatidylserine/PC/GC (40:10:1 mole ratio) the result seems to be formation of coexisting phosphatidylserine-enriched and PC/GC-enriched domains. Hence, in this case at least, a phospholipid and glycolipid have shown the same dynamic response to a lipid-based perturbation.

Order Parameters

A basic similarity between phosphatidylcholines and galactosyl ceramide is clearly seen in lipid bilayer order parameters derived from spin label data. The order parameter, S, has been described in detail by other workers (7, 16–18). Its measurement relies on spin label sensitivity to orientation in the magnetic field and the reflection of this sensitivity in

the EPR spectrum. An S value approaching 1.0 for a fatty acid spin label derivative indicates that the fatty acid backbone region to which the label is attached is in general oriented so that its long axis has a time average orientation ⊥ to the plane of the membrane. S values approaching 0 indicate that the backbone region to which the label is attached is randomly oriented (note however that these statements represent a great oversimplification – see especially discussion of flexibility gradients by McConnell in Ref. 18). In general the degree of fatty acid chain orientation ⊥ to the plane of the membrane decreases towards the center of the bilayer. In the case of (12, 3) spin-labeled lipids S values may be calculated using parameters measured directly from EPR spectra of liposome suspensions. The S values for lipids labeled with the (5, 10) and (1, 14) labels are generally derived from spectra of oriented bilayers on flat surfaces. Figure 2 shows order parameter plots for bilayers of egg PC derived using PC and GC spin labels. Since the spin-labeled lipids were kept at very low concentrations (and are not subject to dramatic clustering) the S values should reflect conditions of the egg PC bilayer immediately surrounding the particular spin label used. Qualitatively the 2 sets of data are very similar. However, quantitatively the egg PC order parameters measured using glycolipid spin labels are consistently higher than those found with phospholipids. Such an observation would be consistent with a tighter, more orderly packing of phospholipids immediately adjacent to glycosphingolipids and/or with glycolipids sitting slightly higher in the membrane (perhaps due to a bulky, H-bonded headgroup) (10). Neither of these possibilities has been ruled out, but we have shown (10) that natural GC

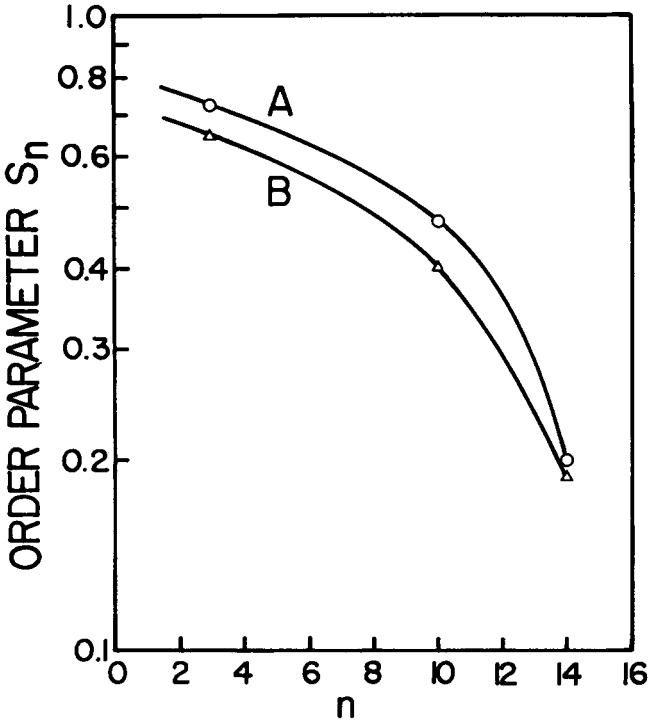


Fig. 2. The order parameter, S, plotted as a function of n, where n is the number of methylene carbons between the spin label ring and the carboxyl function of the fatty acid. The data is for fully hydrated bilayers of egg phosphatidylcholine containing 1% spin-labeled A) galactosyl ceramide and B) phosphatidylcholine. Temperature of all spectra, 23°C. [From Ref. 10 with permission from Elsevier/North-Holland Biomedical Press.]

and beef brain gangliosides confer increased order and rigidity on fluid phospholipid bilayers as would be expected in the case of tighter packing around glycolipids.

Glycolipid Flip-Flop

The flip-flop phenomenon in lipid bilayers was first measured (and found to be slow) using headgroup spin-labeled phospholipids (2). The approach used was to prepare sealed, single bilayer vesicles whose membranes contained a small amount of the spin-labeled lipid. Spin labels in the outer monolayer can be completely reduced by the addition of ascorbate at 0°C, but labels in the inner monolayer are unaffected since the bilayer is impermeable to ascorbate at this temperature. Reduced labels disappear permanently from the EPR spectrum, and peak height can be used as a measure of what fraction remains at the inner surface. Figure 3 shows that the (12, 3) GC spin label may be used for this kind of experiment (see also Ref. 11) but that incubation with cold ascorbate must be carried out for some 75 min to ensure complete reduction of labels in the outer monolayer. The amount of unreduced label (some 31%) is in agreement with that expected from theory for small vesicles (2). Figure 4 shows that if, following ascorbate treatment (see Materials and Methods), the excess ascorbate is removed and the sample incubated at 23°C, there is no appreciable flip-flop of unreduced label to the outer surface over a period of some 5 hr.

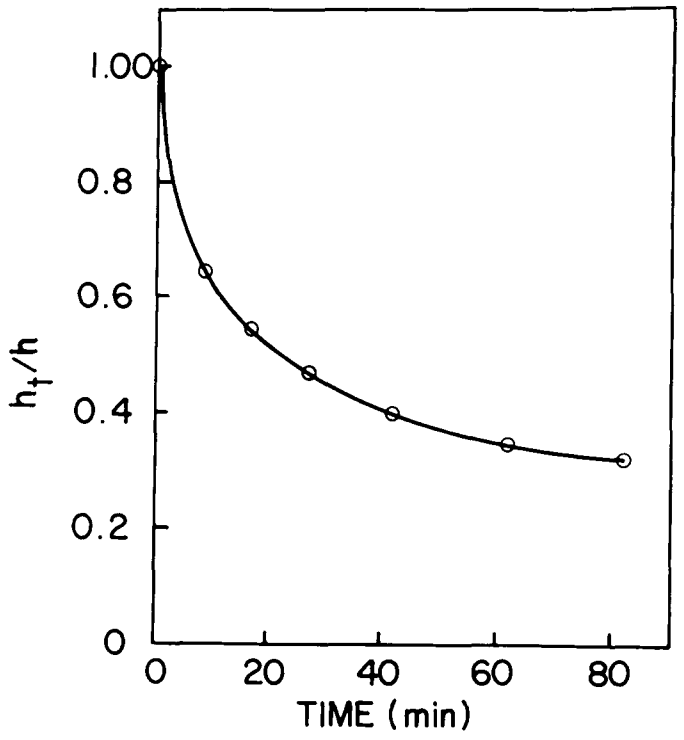


Fig. 3. Reduction kinetics for (12, 3) spin-labeled galactosyl ceramide in sealed lipid bilayer vesicles (~250 Å diameter) at 0°C. The reducing agent is ascorbate added externally and has no access to spin labels occupying the inner monolayer. Lipid mixture is egg phosphatidylcholine containing 5 mol % spin-labeled glycolipid. h_t/h is the ratio of the intensity of the low-field peak at time t to the intensity at time 0.

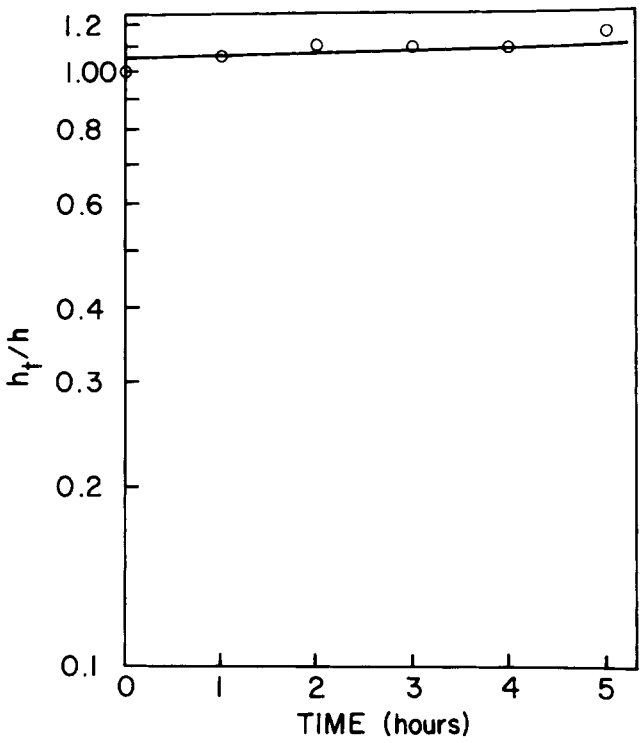


Fig. 4. Fraction of spin-labeled galactosyl ceramide which remains protected at the inner surface of sealed bilayer vesicles following an initial treatment with ascorbate at 0°C, removal of excess ascorbate, and incubation for the times indicated at 23°C. h_t/h is the ratio of the intensity of the low-field 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 is egg phosphatidylcholine containing 4 mol % spin-labeled glycolipid.

In preliminary experiments very similar results have been obtained with the headgroup-labeled gangliosides. In this case the reduction by ascorbate at 0°C is very fast (less than 8 min) leaving a similar proportion of protected label (28%) to that seen in the case of galactosyl ceramide. The conclusion drawn from these experiments is that in very fluid phospholipid bilayers (and hence, by interpolation, in bilayer regions of real membranes) the flip-flop of glycolipids has a half time much longer than 5 hr at 23°C. These experiments put no upper limit on the flip-flop half time.

Ganglioside Behavior and Carbohydrate Headgroup Interactions

So far we have restricted our work with spin-labeled gangliosides to beef brain mixtures labeled in the headgroup itself (see Fig. 1). Such labels should be sensitive to events involving the carbohydrate moieties. Experiments described here were carried out with samples possessing up to 1 nitroxide ring per ganglioside (i.e., 80% or more of the sugar rings are unlabeled). When such labeled ganglioside preparations are studied at 23°C in egg PC bilayers (fluid) or dipalmitoyl PC bilayers (rigid) the spectrum is that of a highly mobile nitroxide (correlation time, $\tau_c \sim 3.8 \times 10^{-10}$ sec in 10 mM phosphate buffer pH 7.0)

(Fig. 5). Hence it seems that, at least at low concentrations in the bilayer, ganglioside headgroups are not highly immobilized and the headgroup behavior is more or less independent of the physical state of the lipid bilayer. This type of labeled ganglioside is probably not very sensitive to spin-exchange broadening resulting from ganglioside clustering since the latter can occur without the nitroxides being brought into close proximity. Hence we have attempted to investigate this phenomenon and that of carbohydrate-carbohydrate headgroup interactions by monitoring spin label mobility (see below).

The existence of surface carbohydrate (whether on proteins or lipids) has been considered potentially important in mediating cell-cell adhesion (19) and association of components within a given cell (20). A possible mechanism for such an involvement would be cooperative attractive forces between carbohydrate residues such as those which are known to exist in certain periodic polysaccharides in solution (21). However, the existence of analogous forces among aperiodic oligosaccharides such as those characteristic of mammalian cell surfaces has not been demonstrated. Such interactions could lead to or stabilize glycolipid and/or glycoprotein clustering such as that which has been postulated to occur in the human erythrocyte membrane (22, 23). In aqueous solvents, the formation of H bonds to water reduces the importance of macromolecule inter- or intramolecular H bonds. But apparently the possibility of forming numerous highly directional H bonds can be very significant – presumably contributing to the extreme insolubility of compounds like cellulose, and of course to the helical nature of DNA and certain polysaccharides such as starch. This

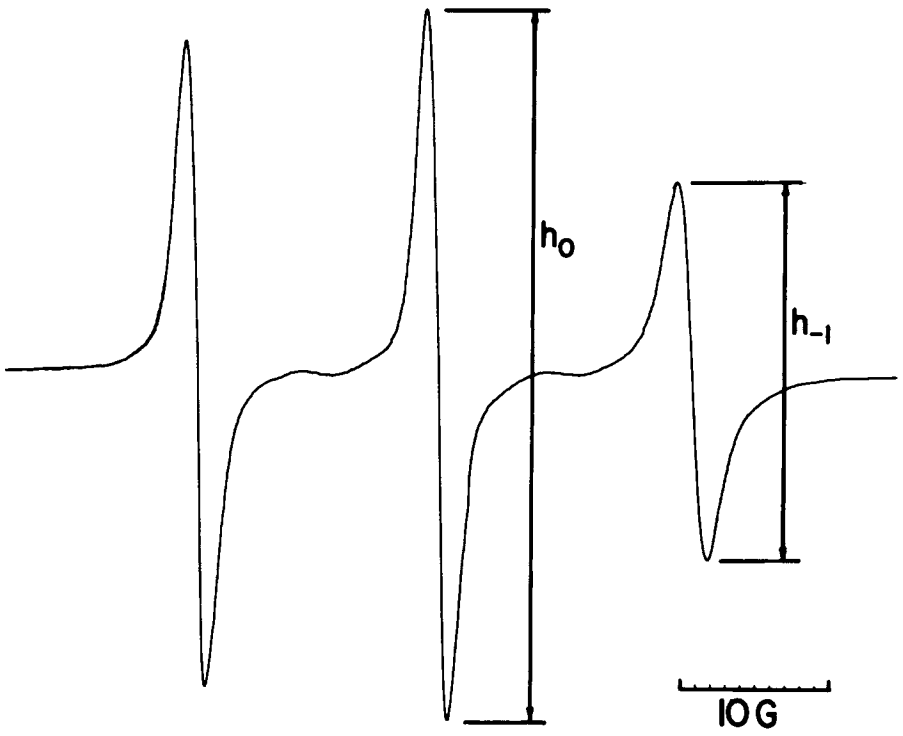


Fig. 5. Typical EPR spectrum of spin-labeled ganglioside (0.45 spin labels per ganglioside) at a concentration of 3.3 mol % in bilayers of egg phosphatidylcholine. The buffer is 10 mM phosphate pH 7.0. The peak heights used for correlation time measurements are indicated (temperature 23°C).

phenomenon should be most significant in cell surface regions where the local carbohydrate concentration is high. In general mammalian cells have much higher surface carbohydrate concentrations than that which exists in our lipid bilayers containing a few mol% ganglioside. We have used a spectral parameter related to the correlation time, τ_c, to examine the effect on ganglioside headgroup mobility of increasing surface carbohydrate concentrations (8; see Fig. 6).

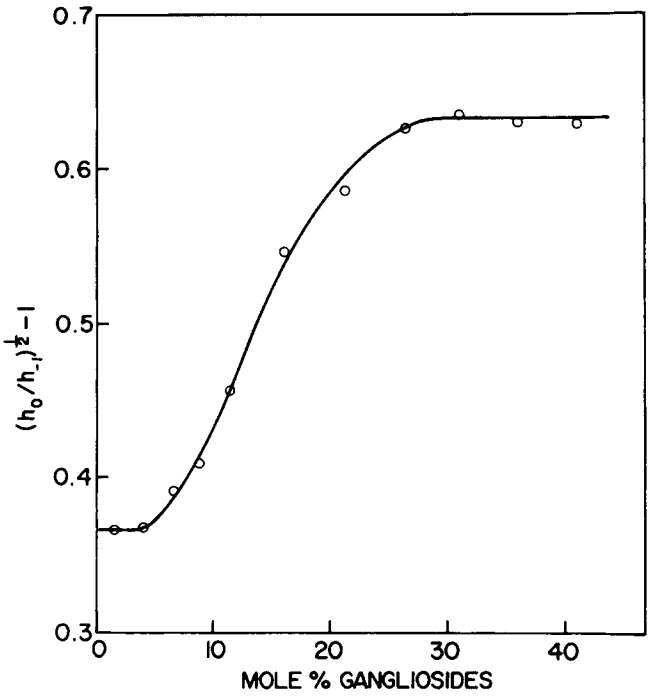


Fig. 6. Effect of increasing amounts of total ganglioside on the oligosaccharide headgroup mobility of spin-labeled gangliosides in fluid lipid vesicles at 23°C. Egg phosphatidylcholine vesicles contain 1.5 mol % spin-labeled ganglioside (0.45 spin labels per ganglioside) and variable amounts of unlabeled ganglioside. Headgroup mobility is inversely related to $[(h_0/h_{-1})^{1/2}-1]$. Vesicles were suspended in 10 mM phosphate pH 7.0.

$$\tau_c = 6.5 \times 10^{-10} W_0 \left[(h_0/h_{-1})^{1/2}-1\right]$$

Where W_0 is the linewidth of the mid-field line, and h_0 and h_{-1} are the heights of the mid- and high-field lines of the spectrum respectively (Fig. 5). This equation assumes rapid, isotropic tumbling of the nitroxide label. For the purposes of the work described here the actual value of τ_c is of little interest except insofar as changes in it may reflect changes in the interactions or mobility of the entire oligosaccharide portion of the ganglioside. In fact we often plot just the fraction, $[(h_0/h_{-1})^{1/2}-1]$ because in our system changes in W_0 are relatively small and the difficulty of accurately measuring W_0 introduces point scatter.

Figure 6 shows that headgroup immobilization increases with added ganglioside according to a sigmoidal curve*, plateauing at about 25% ganglioside in egg PC. Note that this value is for total carbohydrate which in this system is divided between the 2 surfaces, whereas in real cells all carbohydrate is concentrated at 1 surface. The interpretation of this result is not clear-cut (8), but the behavior is consistent with formation of ganglioside-enriched regions in the lipid bilayer as a result of intermolecular attractive forces between the carbohydrate headgroups.

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*When originally published (8) we were uncertain as to the shape of the curve at low ganglioside concentrations and simply drew the best straight line through these points. Further experiments have shown that the sigmoidal shape is genuine.