A GANGLIOSIDE SPIN LABEL: GANGLIOSIDE HEAD GROUP INTERACTIONS

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SUMMARY: A general procedure has been developed for covalent attachment of a nitroxide spin label in the head group region of gangliosides. Gangliosides so labeled and incorporated into lipid bilayer vesicles give a sharp, 3-line spectrum characteristic of a highly mobile structure. The molecular basis of apparent ganglioside-ganglioside head group interaction is briefly discussed.

INTRODUCTION

Gangliosides, a class of glycosphingolipids possessing one or more residues of N-acetyl (or N-glycolyl) neuraminic acid, have become a subject of considerable interest in recent years as important outer surface components of mammalian cell membranes. Their involvement in human sphingolipidoses (1) and their function as cholera toxin receptors (2) are well known. Moreover gangliosides have been implicated in cell-cell adhesion (3), contact inhibition (4), synaptic transmission (5) and cell morphology and differentiation (6). A persistent impediment to understanding the molecular basis of ganglioside function (and indeed that of glycolipids and glycoproteins in general) has been lack of concrete data concerning their physical behaviour in the membrane. In particular little is known about their interaction with one another, with (glyco)proteins, and their distribution response to specific recognition events. As part of a program to investigate the behaviour and interactions of membrane carbohydrate-bearing components, we report here the synthesis of and some preliminary observations on a new type of spin label: head group labeled gangliosides.

Abbreviations: tempophosphate, 4-phospho-2,2,6,6 - tetramethylpiperidine-1-oxyl; tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; tempocholine, N,N-dimethyl-N (1'-oxyl-2',2',6',6'-tetramethyl-4'-piperidinyl)-2-hydroxyethylammonium chloride.

MATERIALS AND METHODS

Beef brain gangliosides were isolated by the method of Kanfer (7), or by a modification thereof in which a silicic acid chromatography step was used to purify crude gangliosides obtained from the initial Folch extraction. Silicic acid columns (Bio Rad 200-325 mesh) were eluted with methanol/chloroform (20-70%) for ganglioside purification. Egg phosphatidylcholine was purchased from Sigma (Type 3-E) and was further purified by chromatography on silicic acid. Tempophoshate (8) and tempocholine chloride (9) were synthesized by conventional literature procedures. Tempol and 2,4,6-triisopropylbenzene-sulfonyl chloride were from Aldrich.

Ganglioside Spin Label Synthesis: 50 mg of beef brain gangliosides and 9 mg of tempophosphate were mixed in 0.5 ml of pyridine (spectral grade, dried over CaH_2) in a small tube and evaporated to dryness under vacuum. This was repeated twice more to ensure the removal of traces of water. 21 mg of triisopropylbenzenesulfonyl chloride in 1.0 ml of dry pyridine was added to the above solution. The reaction mixture was magnetically stirred in a sealed flask containing $CaCl_2$ for 18 hours at 23°C and then the reaction was terminated by addition of 2 ml of distilled water. The mixture was dialysed against 2 changes of distilled water prior to being evaporated to dryness, and the ganglioside fraction purified on a small silicic acid column. Column fractions were monitored by thin layer chromatography on silica gel G (Stahl) eluting with 55/40/2/8 chloroform/methanol/ammonia/water and checked by EPR spectroscopy. Early fractions were contaminated with what appeared to be a biradical of tempophosphate and were discarded. The remaining ganglioside fractions were pooled, dried down and further dialysed against distilled water for 24 hour at 4°C. The purified spin labeled ganglioside was evaporated to dryness, taken up in chloroform/methanol and stored at -20°C. Yield, based on ganglioside starting material, about 60%. The extent of spin labeling of the ganglioside preparation was determined by double integration and comparison with a standard solution of tempocholine chloride. The ratio found was 0.45 spin labels per ganglioside assuming an average ganglioside molecular weight of 1800.

Lipid mixtures were made by dissolving appropriate amounts of each in choloroform/methanol and pumping extensively under vacuum to remove traces of solvent. Dried lipids were suspended in buffer by vortexing. EPR spectra were recorded at 23°C on a Varian E-12 spectrometer interfaced with a Fabritek 1072 Instrument Computer.

RESULTS AND DISCUSSION

Gangliosides are known to exist with their ceramide backbone anchored in the membrane such that the oligosaccharide chain of up to some 7 sugar residues is exposed at the outer surface. The reaction procedure described in the 'Methods' section has the effect of incorporating spin labels into the head group region by formation of phosphate esters. Under the conditions used here there should be a very great preference for esterification (and hence spin labeling) of primary alcohols (10) (of which there is one per sugar residue at the C-6 position). The ratio of label to ganglioside may be controlled, at least to some extent, by varying the reagent ratios. In this work we

have employed low levels of spin label (0.45 labels per ganglioside).

The usefulness of a given spin labeled biological molecule is generally increased by finding highly specific (and known) attachment points for the nitroxide ring. Unfortunately specific attachment of reagents to oligosaccharides is notoriously difficult. In the case reported here the attachment point for the spin probe is only marginally controlled: the reaction used, although a well known one for coupling phosphate to primary alcohols (10,11), cannot be expected to label any particular sugar. It should also be noted that the procedure described adds both another negative charge and a nitroxide-containing ring to the head group region and so cannot be considered totally unperturbing. On the other hand: a) gangliosides possess from one to three negative charges in the head group to start with, and b) there is wide structural and compositional variation in normal ganglioside head groups. Hopefully then the presence of the label will not detract excessively from the observations made, either on the basis of charge or size.

The spectrum obtained from spin labeled ganglioside incorporated into lipid vesicles at low concentration is three sharp lines (see Figure 1) indicating that the label is tumbling rapidly (correlation time τ_c on the order of 3.8×10^{-10} sec*). There is no detectable immobilized component and this would argue against strong intramolecular immobilization of the oligosaccharide head groups (for instance by carbohydrate-carbohydrate hydrogen bonding or ionic interactions).

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* A useful parameter which can be estimated from spectra such as those obtained here is the correlation time, τ_c, for the spin label nitroxide ring. In the case of an isotropically and rapidly ($\tau_c < \sim 10^{-9}$ sec) tumbling label it is possible to arrive at τ_c as follows:

$$\tau_c = 6.5 \times 10^{-10} W_o [(h_o/h_{-1})^{1/2}-1]$$

where W_o is the line width of the mid-field line and h_o and h_{-1} are the heights of the mid-and high-field lines of the spectrum respectively (see Fig. 1) (12).

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, $[(h_o/h_{-1})^{1/2}-1]$ because in our system changes in W_o are relatively small and the difficulty of accurately measuring W_o introduces point scatter.

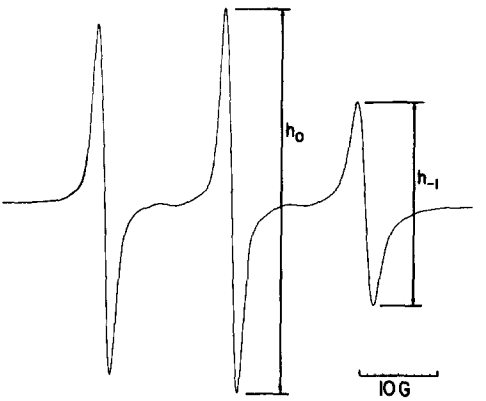

FIGURE 1

Typical EPR spectrum of spin labeled ganglioside (0.45 spin labels per ganglioside) in lipid bilayers. The sample is 2 mg of egg phosphatidylcholine containing 3.3 mole % ganglioside in 50 µl of 10 mM phosphate buffer pH 7.0 (temperature 22°C).

One of our primary research objectives is to answer questions regarding the behaviour and interactions of membrane carbohydrate-bearing components with one another and with other membrane components. To this end we earlier reported the synthesis of and studies with, spin labeled derivatives of the neutral glycolipid, galactosyl ceramide (13,14). So far our experience has been that neutral glycolipids behave in a fashion similar to phospholipids except for a measurable tendency to rigidify fluid lipid bilayers. With the labels reported in this article one should be able to test various hypotheses concerning ganglioside behaviour (and indeed that of outer surface oligosaccharides in general). One of the first questions to which we have addressed ourselves is the existence (or nonexistence) of oligosaccharide intermolecular attractive forces which might be important in phenomena such as cell-cell adhesion, membrane component distribution and the cryptic nature of certain cell surface recognition sites (e.g. ref 3,16).

Figure 2 demonstrates that gangliosides certainly do interact with one another in fluid lipid bilayers. The head group region of spin labeled gangliosides is increasingly immobilized by incorporation of unlabeled gang-

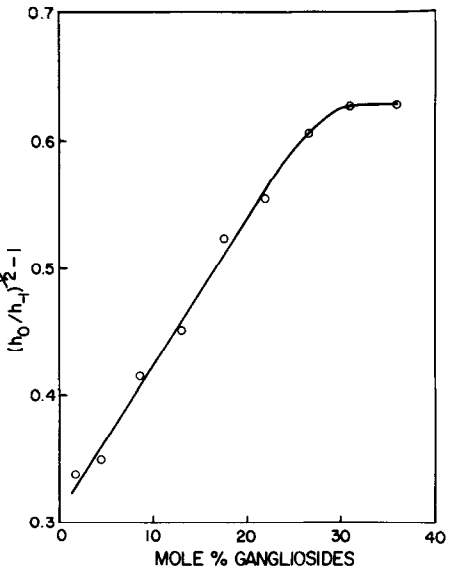

FIGURE 2

Effect of increasing amounts of total ganglioside on the oligosaccharide head group mobility of spin labeled gangliosides in fluid lipid vesicles at 22°C. Egg phosphatidylcholine vesicles contain 1.7 mole % spin labeled ganglioside and variable amounts of unlabeled ganglioside. Head group mobility is inversely related to $[(h_o/h_{-1})^{1/2} -1]$.

lioside up to a ratio of 28 mole %. At first sight the effect seen could be due simply to rigidification of the lipid bilayers by increasing glycolipid concentration (it has recently been shown by ourselves (14) and by Tinker et al (16) that glycolipids do have this effect). However this explanation of the data can be ruled out by comparing τ_c for 1.7% labeled ganglioside in a very fluid lipid (dioleoyl phosphatidylcholine at 23°C) to that in a rigid lipid (dipalmitoyl phosphatidylcholine) at the same temperature. The rigidity difference between these two lipids (as indicated by spin label-derived order parameters) is some three times that brought about by addition of 36 mole % ganglioside to egg phosphatidylcholine, yet it causes an increase in $[(h_o/h_{-1})^{1/2}-1]$ for the ganglioside spin label of only 20%. On this basis ganglioside-induced bilayer rigidification can account for only a small portion (less than 7%) of the increase observed in $[(h_o/h_{-1})^{1/2}-1]$ when the ganglioside

concentration in egg phosphatidylcholine is increased from 0 to 36%. The ganglioside-ganglioside interaction leading to head group immobilization could be attributed to a simple viscosity effect (i.e. increasing surface concentrations of mobile carbohydrate head groups) and/or to attractive forces between oligosaccharide chains. At present we are trying to determine the relative contributions of these two factors. It is difficult to accurately estimate the viscosity effect which would be generated immediately adjacent to a lipid bilayer surface by increasing concentrations of ganglioside carbohydrate head groups (one can probably assume maintenance of the bilayer array up to some 50 mole % (16,17)). However we have investigated the effect of viscosity on τ_c of free tempophosphate: τ_c increases roughly exponentially with increasing sucrose concentration over a wide range (unpublished results). This is quite different from the ganglioside head group behaviour seen in Figure 2 in which τ_c increases linearly and then steeply levels off. Such behaviour is however consistent with spontaneous formation of ganglioside-enriched regions in the lipid bilayer as a result of intermolecular attractive forces between oligosaccharide chains. In this case increasing ganglioside concentration would be expected to lead to head group immobilization up to a limiting value of τ_c.

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