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The Behaviour Of Glycosphingolipids Under A Variety Of Structural And Environmental Influences

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THE BEHAVIOUR OF GLYCOSPHINGOLIPIDS UNDER A VARIETY OF STRUCTURAL AND ENVIRONMENTAL INFLUENCES

by

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ABSTRACT

The influence of naturally occurring glycosphingolipid (GSL) structural variations as modulators of glycolipid organization and dynamics was considered by wideline ^{2}H-NMR in bilayer model membranes. Non-perturbing deuterium probes were placed at selected locations within polar and hydrophobic regions. A variety of environmental influences, which have been widely considered as major determinants of GSL function as recognition sites and structural elements, was examined.

GSL oligosaccharide orientation and motional order were monitored while systematically altering primary GSL structure changes and membrane environment. Each glycolipid, as a minor membrane component, demonstrated clear evidence of preferred average oligosaccharide orientation. Strikingly, the conformation and motional order of all the GSL headgroups monitored in fluid membranes were only modestly influenced by factors tested, including natural and artificially introduced variations to the GSL hydrophobic region, membrane fluidity, temperature, and presence of cholesterol or the NANA residue of gm_1. High Ca^{2+} concentrations produced spectral changes in asialo-gm_1 (neutral GSL) and gm_1 (charged), which may reflect generalized ion binding to the membrane.

Measurement of spectra from deuterons located in the

hydrophobic region of the GSL revealed the effects of fatty acid variation. Results of GSL fatty acid hydroxylation (D- and L-isomers) overall suggested that one significant aspect of the α-hydroxy group was to interfere with glycolipid packing amongst host phospholipids in the upper portion of the acyl chains. For the D-α-hydroxy stereoisomer, there was some evidence of minor strengthening of interlipid interaction near the membrane surface. Effects of GSL unsaturation proved to be very similar to published data dealing with glycerol based lipids in cell and model membranes. Phase diagrams of N-stearoyl (C-18) and N-lignoceroyl (C-24) galactosylceramide in SOPC were produced. The fluidus curves of both systems were virtually superimposable while a major difference was seen in ordered phase behaviour. At low concentrations, the phase behaviour of the C-18 and C-24 GalCer systems were very similar in both fluid and gel phases. Unique behaviour was observed for the omega end of very long GSL fatty acids in fluid phospholipid membranes.

DEDICATION

I wish to dedicate this thesis to my parents and girlfriend, Carol, for their love, support and strength that I received from them over the years.

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TABLE OF CONTENTS

Page

CERTIFICATE OF EXAMINATION ii
ABSTRACT iii
DEDICATION v
ACKNOWLEDGEMENTS vi
TABLE OF CONTENTS vii
LIST OF TABLES x
LIST OF FIGURES xi
ABBREVIATIONS xiii

INTRODUCTION 1

CHAPTER 1 - EFFECTS OF FATTY ACID α-HYDROXYLATION ON GLYCOSPHINGOLIPID PROPERTIES 9

1.1 INTRODUCTION 1

1.2 MATERIALS AND METHODS 11
1.2.1 Source of materials 11
1.2.2 Synthesis of D- and L-α-OH stearoyl GalCer . . 12
1.2.3 Preparation of samples for ^{2}H-NMR 16
1.2.4 Acquisition of spectra 16

1.3 RESULTS AND DISCUSSION 17
1.3.1 General features 24
1.3.2 Contrasting α-hydroxy with the non- hydroxy analogue in phosphatidylcholine bilayers 24

1.4 CONCLUSIONS 28

CHAPTER 2 - THE INFLUENCE OF AN UNSATURATED FATTY ACID ON THE BEHAVIOUR OF A GLYCOSPHINGOLIPID IN PHOSPHATIDYLCHOLINE BILAYERS 30

2.1 INTRODUCTION 30

2.2 MATERIALS AND METHODS 32
2.2.1 Source of materials 32

2.2.2 Sample preparation 32
2.2.3 Acquisition of spectra 33

2.3 RESULTS AND DISCUSSION 36
2.3.1 In General 36
2.3.2 Contrasting deuterated N-(oleoyl)-GalCer with glycerolipids - not too much contrast! 40
2.3.3 Comparing unsaturated GalCer with saturated GalCer 48
2.3.4 Unsaturated GalCer in an unsaturated matrix . 49

2.4 CONCLUSIONS 50

CHAPTER 3 - GLYCOSPHINGOLIPID PHASE BEHAVIOUR AND ACYL CHAIN ORDER 51

3.1 INTRODUCTION 51

3.2 MATERIALS AND METHODS 55
3.2.1 Source of materials - synthesis of N-(lignoceroyl-d_{47}) and N-(stearoyl-d_{35}) galactosylceramide 55
3.22 Synthesis of omega d_7 lignoceric acid 56
3.23 Sample preparation and acquisition of spectra 57

3.3 RESULTS AND DISCUSSION 61
3.3.1 N-lignoceroyl GalCer spectra 62
3.3.2 N-stearoyl GalCer spectra 83

3.4 CONCLUSIONS 85

CHAPTER 4 - THE INFLUENCE OF THE CERAMIDE FATTY ACID STRUCTURE ON THE BEHAVIOUR OF THE CARBOHYDRATE HEADGROUP OF GALCER 87

4.1 INTRODUCTION 87

4.2 MATERIALS AND METHODS 90
4.2.1 Source of Materials 90
4.2.2 Synthesis of galactosylceramide species 91
4.2.3 Deuteration of galactosylceramides 92
4.2.4 Preparation of samples for ^{2}H-NMR. 93
4.2.5 Acquisition of spectra 94

4.2.6 Freeze Fracture 94

4.3 RESULTS AND DISCUSSION 95
4.3.1 Background 95
4.3.2 In general 103
4.3.3 Comparison between samples and temperatures 103
4.3.3.1 GalCer species share some behavioural characteristics 103
4.3.3.2 The effect of temperature on headgroup orientation 106
4.3.3.3 Phase separation at lower temperatures . . . 109

4.4 CONCLUSIONS 114

CHAPTER 5 - THE EFFECT OF NATURALLY OCCURRING MEMBRANE ATTRIBUTES ON COMPLEX GSL OLIGOSACCHARIDE BEHAVIOUR 115

5.1 INTRODUCTION 115

5.2 MATERIALS AND METHODS 120

5.3 RESULTS 122
5.3.1 General features observed in spectra of GSLs deuterated in specific sugar exocyclic hydroxymethyl groups 125
5.3.2 General features observed in spectra of GSLs deuterated in amino-sugar acetate groups . 134
5.3.3 Effect of GSL hydrophobic portion 138
5.3.4 Effect of cholesterol 143
5.3.5 Effect of temperature/membrane fluidity . . . 144
5.3.6 Effect of desialation 145
5.3.7 Other factors 146

5.4 DISCUSSION 147

5.5 CONCLUSIONS 160

REFERENCES 163

VITAE 182

LIST OF TABLES

Table	Description	Page
1.1	Quadrupolar splittings for deuterated GSLs in DMPC	22
2.1	Literature and experimental values for unsaturated glycerolipids and GSLs respectively	47
4.1	Acyl chain dependencies of quadrupolar splittings for headgroup labelled galcer	108
5.1	Quadrupolar splittings for hydroxymethyl labelled GSLs	128
5.2	Quadrupolar splittings for acetate deuterated GSLs	129

LIST OF FIGURES

Figure	Description	Page
1.1	Chemical structures of GalCer	14
1.2	Spectra for deuterated GalCer (30°C & 50°C)	20
1.3	Spectra for deuterated GalCer (9°C & 19°C)	26
2.1	Spectra for pure C-18:1 GalCer	35
2.2	Spectra for C-18:1 GalCer in DMPC	39
2.3	Spectra of various deuterated lipids	43
2.4	Order parameter plot of C-18:1 GalCer vs glycerolipid data	45
3.1	Spectra of C-24 GalCer in SOPC	64
3.2	Spectra of C-18 GalCer in SOPC	66
3.3	Spectra of C-24 (d_7 and d_{47}) GalCer in SOPC	68
3.4	Spectra for GalCer -pure C-24 and in SOPC	72
3.5	First spectral moment plot of C-24 GalCer in SOPC	76
3.6	Phase diagram for C-24 and C-18 GalCer in SOPC	80
3.7	First spectral moment plot of C-18 GalCer in SOPC	82

4.1	Chemical Structures of GSLs studied	98
4.2	Spectra of GalCer differing in fatty acid in POPC 65°C	100
4.3	Spectra of GalCer differing in fatty acid in POPC 40°C	102
4.4	Freeze fracture photograph of GalCer systems	112
5.1	Structures of complex GSLs	118
5.2	Selected spectra from complex GSL systems	124
5.3	Selected spectra from complex GSL systems	137
5.4	Selected spectra from complex GSL systems	141

ABBREVIATIONS

C-18	N-stearoyl galactosylceramide
C-24	N-lignoceroyl galactosylceramide
C-18:1	N-oleoyl galactosylceramide
-CDHOH	C_6 hydroxymethyl galactose or N-acetylgalactosamine residue deuteration
D-α-OH	N-(D-α-hydroxy)-stearoyl galactosylceramide
*d*Gal	C_6 hydroxymethyl galactose residue deuteration
*d*GalNac	N-acetylgalactosamine residue deuteration
DMPC	dimyristoyl phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
DSC	differential scanning calorimetry
DSPC	distearoyl phosphatidylcholine
EDTA	ethylenediamine tetraacetic acid
EPR	electron paramagnetic resonance
Gal	galactose
GalCer	galactosylceramide or cerebroside
GalNac	N-acetyl galactosamine
GlcCer	glucosyl ceramide
GSL	glycosphingolipid

^2H	deuterium
HOD	monodeuterated water
L-α-OH	N-(L-α-hydroxy)-stearoylgalactosylceramide
lysoGalCer	galactosylceramide without the N-acyl fatty acid
NANA	neuraminic acid
NMR	nuclear magnetic resonance
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
SOPC	1-stearoyl-2-oleoyl phosphatidylcholine
sulphatide	sulphated galactosylceramide

INTRODUCTION

Glycosphingolipids (GSLs) are a class of carbohydrate bearing lipids present on the outer surface of eucaryote cells (Thompson and Tillack, 1985, Curatolo, 1987a; 1987b). Superficially, their structure is similar to phospholipids in that there are 2 alkyl chains and a polar headgroup. GSLs consist of a carbohydrate chain attached in glycosidic linkage to a ceramide backbone. This non-glycerol backbone has the capacity for donor as well as acceptor H-bonding. The ceramide portion of the GSL is composed of a sphingosine base bound in amide linkage to a fatty acid. There is a great deal of variety in both the carbohydrate and fatty acid structures. The sphingosine portion varies to a much lesser extent. The fatty acid can range in length from 18 to 28 carbons thus being generally longer than the 16-18 carbon fatty acids seen for phospholipids. In addition, the GSL fatty acid is often hydroxylated at C_2, and can be unsaturated. GSLs are a focus of great interest because of their proposed roles in recognition, attachment, and membrane architecture. Their compositional change with differentiation, cell cycle, transformation and oncogenesis is also a major area of GSL investigation (Hakomori, 1986; Curatolo, 1987a; 1987b).

The sugar directly attached to the ceramide moiety is either galactose or glucose. This carbohydrate portion, or headgroup, can be more complex

than this to include a number of sugar residues (Thompson and Tillack, 1985). The structure of the GSL carbohydrate headgroup determines their classification; i) simple neutral (eg. cerebroside also known as galactosylceramide) ii) complex neutral (eg. globoside) iii) charged complex or gangliosides (eg. gm_1) and iv) sulphatide (eg. cerebroside sulphate also known as sulphatide). The role of GSLs as specific recognition sites has focused interest on the conformation and arrangement of their oligosaccharide chains. Very little is known about the membrane surface behaviour of carbohydrate headgroups that are more complex than one sugar. Two major facets in the control of oligosaccharide arrangement and dynamics are primary sequence and remote effects (inter- or intramolecular interactions). Several studies using 2-dimensional high resolution NMR have examined oligosaccharide arrangement in solutions of intact gangliosides (Scarsdale et al., 1990; Acquotti et al., 1990) and globoside (Maggio et al., 1990; Scarsdale et al., 1986). These studies discussed the general expectation that inter- and intramolecular effects on oligosaccharide chains would be important. Variables such as receptor primary structure, spacial constraints, and proximity to a membrane surface are sources of remote effects.

It has been widely considered that the nature of the GSL hydrophobic portion modulates its functions. The suggested mechanisms include control

of lateral mobility, distribution, carbohydrate orientation/accessibility and lipid dynamics (Hakomori, 1986; Curatolo, 1987a; 1987b; Thompson and Tillack, 1985; Grant, 1984) In particular, fatty acid hydroxylation (Kannagi et al., 1983, Curatolo and Jungalwala, 1985; Maggio et al., 1985; Sampio et al., 1986; Boggs et al., 1988; Johnston and Chapman, 1988), chain length (Alving et al., 1980; Crook et al., 1986) and unsaturation (Ali et al., 1989; Reed and Shipley, 1989) have been suggested to be sources of such effects.

Pure hydrated GSL systems have been studied in detail and have provided a foundation for further investigation of their physical properties in more complex settings. Extrapolation of the results from investigations of pure systems to the biological setting has been criticized because of the simplicity of these pure artificial systems. Further study has been done in more complex and biologically relevant systems using DSC, X-ray diffraction, fluorescence and antibodies. We have observed repeatedly that forces widely presumed to be important from studies of simpler systems had much less impact when dispersed as minor components in fluid membranes.

The focus of this thesis is the study of GSL behaviour in phospholipid bilayers, examining the influence of structural diversity and membrane environments. The structural diversity of the GSL suggests a diversity of function and behaviour. The effects of fatty acid chain features and

carbohydrate structure are examined. The second aspect of investigation involved the influence of the system composition including the membrane matrix composition and factors such as temperature, calcium and proteins. As already mentioned, both the structural and environmental variables of glycolipid systems have been shown to importantly modulate both the structural and receptor properties of the GSL. (For reviews see Hakomori, 1981; Thompson and Tillack, 1985; Curatolo, 1987a; Grant, 1987). An example of a structural role would be in the myelin membrane which has a high content of GalCer (24%) and sulphatide (4%) (Curatolo, 1987a). The high concentration of GalCer has been suggested to provide structural integrity and insulation. As a receptor, GSLs have been found to interact with viruses, bacteria, lectins and toxins (Curatolo, 1987a). Aberrant expression of gangliosides has been observed on the surface of various cancers. It has been suggested that the immune response to specific gangliosides on tumour cells is vital in the modulation of tumour cell growth. One of these gangliosides, gm_3 (a sialic acid containing glycosphingolipid), was found to be antigenically active in neoplastic cell lines in culture and in tissue specimens such as melanoma, colon, breast and lung cancer. Interestingly though, the antibody used in these studies was not reactive towards normal cells in culture or biopsy tissue despite the fact that these normal tissues also possess gm_3. This phenomenon has been suggested to be related to gm_3's

density on the cell membrane surface (Hoon et al., 1993) but very little is known about the details of such phenomena. Hence, the question arises of which property (eg. physical arrangement or structure etc.), is responsible for distinguishing the cancer cell antigen, gm_3, from that of the normal cell. This last phenomenon has been termed receptor "crypticity" which refers to the modulation of GSL antigenicity by intermolecular interactions in the membrane (Hakomori, 1981).

Wideline ^{2}H-NMR is the experimental technique used to study various GSL systems in this thesis. Labelling of the molecule of interest involves the replacement of selected protons with deuterium. Deuterium has a van der Waal radius identical to that of the proton therefore no perturbation to the system is introduced. The low natural abundance of deuterium allows the selective enrichment of this probe at the molecular site of interest thus permitting the direct monitoring of the behaviour of the labelled site.

Deuterium is a quadrupolar nucleus with a spin number I=1. The presence of an external magnetic field eliminates the degeneracy of the nuclear energy levels resulting in two allowed transitions of equal energy which are further differentiated by the electric quadrupole. The result is a NMR spectrum with two peaks. The splitting of these two peaks is referred to as the quadrupolar splitting ($\Delta\nu_Q$) which is measured in frequency units. Anisotropic motion of the labelled system leads to the incomplete averaging

of the residual quadrupolar interactions, giving rise to a quadrupolar splitting. The frequency separation of the splittings are strongly influenced by the orientation and motion of the labelled entity. The quadrupole splitting for rapid axial diffusion may be described by:

$$\Delta \nu_Q = 3/2\ e^2Qq/h\ (S_{CD})\tfrac{1}{2}(3\cos^2\delta - 1) \qquad (1)$$

where e^2Qq/h is the quadrupole coupling constant (Seelig, 1977), S_{CD} is the order parameter of the C-^{2}H bond vector and δ is the angle between the axis of motional averaging (generally the bilayer normal for membrane lipids) and the magnetic field. S_{CD} provides a measure of time averaged angular fluctuations of tne C-^{2}H bond vector with respect to the bilayer normal. For an unoriented liposomal system, the type of spectrum observed is :eferred to as a powder spectrum. Since the intensity of the 90° orientation ($\delta = 90°$) from this spectrum is greatest, the splitting resulting from the 90° orientation is measured. Therefore equation (1) simplifies to:

$$\Delta \nu_{Qpowder} = 3/2\ e^2Qq/h\ (S_{CD})(\tfrac{1}{2}) \qquad (2)$$

For tne consideration of the spacial orientation of the GSL C-^{2}H bonds $\Delta \nu_Q$ can be described as;

$$\Delta \nu_Q = 3/8\ e^2Qq/h\ (S_{mol})(3\cos^2\Theta - 1) \qquad (3)$$

where Θ is the angle between the C-^{2}H bond and the axis of motional averaging. S_{mol} is the molecular order parameter (assuming axially symmetric order) describing the orientational fluctuations of the molecule relative to the bilayer normal.

In addition to the spectral splitting derived from a sample undergoing rapid axial diffusion, the actual shape of the spectrum also gives information about labelled lipid dynamics. An axially symmetric powder spectrum of a lipid sample denotes a disordered fluid phase while a more broad rounded spectrum, which shows no splittings, is evidence of a highly ordered or gel phase (for detail regarding gel phase spectra please see Speyer et al., 1989). These two spectral features would overlap from a system that has both significant gel and liquid crystalline properties.

In this thesis, I have covered the investigation of the effects of fatty acid chain length, C_2-hydroxylation and unsaturation on GSL characteristics in a membrane environment. GalCer, though simple in structure, is a widely studied GSL that has been the subject of the major classic experiments in the literature on recognition site modulation and crypticity. In the present investigations, GalCer was used as one of the models of a GSL receptor and structural entity. It was felt that this molecule should be sensitive to

membrane conditions as well as structural aspects because of its close proximity to the membrane surface. More attention is given to the C-24 and C-18 GalCer/SOPC systems with the production of a phase diagram. The headgroups of the more complex GSLs, gm_1, globosid $_3$ and asialo-gm_1 (see Chapter 5, Fig.5.1) are also studied under a variety of conditions. These aspects are dissected individually in the following chapters.

CHAPTER 1

EFFECTS OF FATTY ACID α-HYDROXYLATION ON GLYCOSPHINGOLIPID PROPERTIES

1.1 INTRODUCTION

Glycosphingolipids exhibit great variety in structure. One important variable is the fatty acid on the ceramide moiety. GalCer from beef brain is a system that has been widely studied as an important example of glycosphingolipids. Hydroxylation of the C-2 or α position of the fatty acid in naturally occurring GalCer from beef brain is very common, comprising approximately 60% (Pascher, 1976). This is in contrast to phospholipids which do not possess hydroxylated fatty acids. There has been a great deal of speculation regarding the effect of hydroxylation on glycolipid characteristics in cell membranes. As mentioned previously, GalCer is a major component of the myelin membrane. In addition to added membrane stability through hydrogen bonding (Pascher, 1976, Neuringer et al., 1979), alpha-hydroxylation of the fatty acid on cerebrosides has been suggested to be of importance in nerve conduction. Strikingly, it has been observed that the nerve conduction velocity in *Caudata* species having no α-hydroxylated fatty acids in their myelin cerebrosides was significantly

reduced (Ki et al., 1985). It has been widely considered that fatty acid α-hydroxylation is a factor in the behaviour of a GSL through the modulation of GSL "crypticity" (Kannagi et al., 1983; Lampio et al., 1986). Part of this argument revolves around the concept of intermolecular hydrogen bonding, which some claim as a significant force responsible for the properties of GSLs (Curatolo, 1987a; Curatolo, 1987b).

Physical studies of the pure hydroxylated fraction of GalCer have been done. It has been shown to form a more disordered gel phase in comparison to the non-hydroxylated fraction. Thus, rather than being a major attractive force, the hydroxyl group, on the fatty acid moiety of GalCer, has also been suggested to be spatially perturbing - thus disrupting tight acyl chain packing characteristic of a gel phase (Bunow and Levin, 1980; Curatolo and Jungalwala, 1985; Maggio et al., 1985). In contrast, studies of pure sulphated GalCer, possessing hydroxylated fatty acids, showed that it had a higher phase transition temperature and enthalpy for the gel-to-liquid crystalline transition as compared to the non-hydroxylated fraction (Boggs et al., 1988). It was suggested that the repulsive effects from the sulphate on the galactose sugar ring may be moderated by a hydrogen bonding network which includes the α-hydroxyl group. Differential scanning calorimetric evidence from a study examining a phospholipid/cerebroside system suggested that α-hydroxylated cerebrosides were more miscible with

phospholipids than the non-hydroxylated counterpart. This was considered to be a result of weaker GSL-GSL attraction in the phospholipid matrix (Johnston and Chapman, 1988).

In the present study, the effects of fatty acid hydroxylation on GSL arrangement and dynamics, at low concentration in DMPC phospholipid membranes, were examined using ^{2}H-NMR. Three GalCer species were studied - both of the D and L-α-OH isomers as well as the non-hydroxylated N-stearoyl fatty acid derivative (see Fig. 1.1). The fatty acids were perdeuterated thus allowing direct monitoring of the behaviour of this hydrophobic region within the membrane.

1.2 MATERIALS AND METHODS

1.2.1 *Source of materials*

1,2-dimyristoyl phosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids, Birmingham, AL; and was used without further purification. Galactosyl ceramide (GalCer) was isolated from beef brain, using the non-polar residue of a Folch extraction (Folch et al., 1957), or purchased from Avanti Polar Lipids, Birmingham, AL. The former material was applied to a column of Bio Rad silicic acid 200-400 mesh, and eluted with a gradient of methanol in chloroform. Isolated GalCer co-chromatographed with known

material on Merck silica gel 60 thin layer chromatography plates eluted with 65:15 $CHCl_3/CH_3OH$ and developed with sulphuric acid/ethanol.

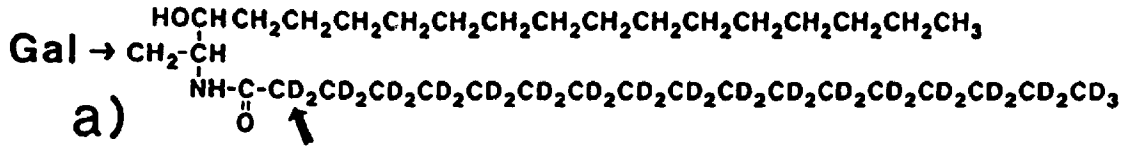
1.2.2 *Synthesis of D- and L-α-OH stearoyl GalCer*

Several attempts were made at synthesizing α-OH stearoyl GalCer. One attempt involved the manufacture of α-hydroxy stearic acid using PBr_3 and Br_2 (Hell-Volhard-Zelinsky reaction - Migrdichian, 1957) followed by the introduction of the hydroxyl group via base hydrolysis of the resulting α-bromo derivative. The α-hydroxylated fatty acid was then coupled to lyso-GalCer using dicyclohexylcarbodiimide (DCC) in pyrimidine. In addition, the reaction of the N-succinimidyl ester form of α-OH stearic acid with lyso-GalCer was attempted. Both of these attempts at making α-OH GalCer, as judged by TLC, were extremely low in yield and were accompanied with many side products. These methods were therefore abandoned. The following synthesis was then devised and proved to be of very high yield with a minimum of side products. An added benefit was that the L-α-OH product, also formed, permitted the study of the importance of the C_2 α-OH configuration.

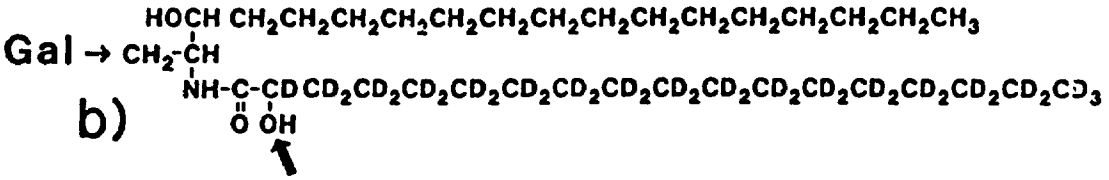
Preparation of α-acetoxy (perdeuterated) stearic acids was via the corresponding α-bromo intermediates. The α-bromo intermediates were generated by reacting octadecanoic acid [d_{35}] (MSD Isotopes Ltd) with

Figure 1.1

Chemical structures of the deuterated glycolipids, N-stearoyl [d_{35}] GalCer (a), and N-α-OH stearoyl[d_{34}] GalCer (b).



a)



b)

liquid bromine in the presence of PCl_3, followed by hydrolysis of the acid chloride product (Allen and Kalm, 1967). These were in turn converted to the corresponding α-acetoxy esters by refluxing with anhydrous sodium acetate in glacial acetic acid (Guest, 1947). The mixture of stearic acid α-acetoxy esters was purified on a silicic acid column eluted with $CHCl_3$, and converted to the acid chloride form by refluxing with excess $SOCl_2$ (Kopaczyk and Radin, 1965). These reactions were followed by TLC on silicic acid plates eluted with hexane/diethyl ether/formic acid (70/30/1). The α-acetoxy ester was identified by ^1H NMR in C^2HCl_3. Probe-labelled D- and L-α-OH GalCer were synthesized by coupling the above α-acetoxy derivatives of the deuterated fatty acid chlorides with lyso-GalCer, followed by hydrolysis of the acetate group (Pascher, 1974). Lyso-GalCer (i.e. with fatty acid removed) was produced from GalCer by hydrolysis in stirred methanolic KOH at 97°C in a sealed glass culture tube (Neuenhofer et al., 1985) or in refluxing butanolic KOH (Taketomi and Yamakawa, 1963) and was ninhydrin positive. The D- and L-α-OH stereoisomers of the GSL were separated using a silicic acid column eluted with a $CHCl_3/CH_3OH$ gradient. The L- isomer ran significantly faster in the solvent system described (Pascher, 1974). Semi-synthetic non-hydroxy and D-α-hydroxy GalCer behaved similarly, but not identically, on TLC to natural beef brain GalCer non-hydroxy and D-α-hydroxy fractions respectively: the semi-synthetic species ran slightly slower than their

corresponding natural mixtures, which included longer fatty acids, as reported previously (Karlsson and Pascher, 1971).

1.2.3 *Preparation of samples for ^{2}H-NMR*

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio (10 mol % glycolipid) in 3:1 $CHCl_3/CH_3OH$, and removing the solvent under a N_2 atmosphere. Resultant films were further dried under vacuum (rotary pump) for 3hr at 22°C. Liposomes were generated by hydration of such films with deuterium depleted water (MSD Isotopes Ltd). Samples were lyophilized three times from a volume of 200 µl of deuterium depleted water after which the hydrated samples were subjected to eight freeze-thaw cycles following the final hydration step. Total lipid used per sample was typically 60-90 mg, and the total volume was 200-300 µl. All samples were incubated 10°C above the transition temperature of the host matrix for 15 min to assure diffusional equilibrium within the bilayer.

1.2.4 *Acquisition of spectra*

^{2}H NMR spectra were acquired at 30.7 MHz on a "home-built" spectrometer operated by a Nicolet 1280 computer (Singh et al., 1992a). The sample was enclosed in a glass dewar and the temperature was

electronically regulated to within ± 0.5 degrees. The spectra were recorded using the quadrupolar echo pulse sequence (Davis et al., 1976) with full phase cycling (Perly et al., 1985) and quadrature detection. The $\pi/2$ pulse length varied from 2.2 to 2.5 μs with the 5 mm solenoid coil, and 5-6 μs with the 10 mm coil. Pulse spacing was 60 μs, and recycle time was 800 ms. Spectra were not folded about the Larmor frequency. 90° oriented sample ("depaked") spectra were calculated from the powder spectra as described previously (Rance and Byrd, 1983).

^{31}P NMR spectra were acquired at 121.5 MHz on a Bruker MSL-300 spectrometer. The spectra were recorded using a Hahn echo pulse sequence (Rance and Byrd, 1983) with Waltz decoupling (gated on during acquisition). The $\pi/2$ pulse length was 4.0 μs (10 mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 7.5 ms.

1.3 RESULTS AND DISCUSSION

1.3.1 *General features*

Since the fatty acid chain of GalCer was perdeuterated in the various GalCer species studied, its behaviour could be monitored directly. N-stearoyl GalCer was compared to the L- and D-α-OH GalCer analogues.

Both the hydroxy and nonhydroxy species were of the same chain length and were incorporated at 10 mol% in the phosphatidylcholine host matrix. This allowed the effect of the variable between the systems, the α-OH substituent, to be studied and its contribution to the characteristics of the hydrophobic region of the GalCer molecule assessed.

^{2}H-NMR spectra of all three fatty acid perdeuterated GalCer species are shown in Fig. 1.2. Each one was at 10 mol% in DMPC bilayers at 30°C and 50°C. The corresponding 90° oriented sample (depaked) spectrum is shown to the right of each spectrum. The phase transition temperature of pure DMPC is 23°C (Shimshick and McConnell, 1973). Despite the fact that the spectra taken were well below the phase transition of the deuterium labelled species (70°C and 83°C for D-α-OH and N-stearoyl GalCer respectively (Curatolo and Jungalwala, 1985), they had characteristics of c liquid crystalline phase. There was no evidence of a gel phase present. This suggested uniform dispersion of the GalCer species throughout the DMPC host matrix. ^{31}P-NMR of the same systems displayed an axially symmetric liquid crystalline lineshape with a $\Delta\sigma$ of approx. 40ppm (Seelig, 1978).

Fatty acid perdeuterated phosphatidylcholine species have been studied (Davis, 1983). Many of the features of the liquid crystalline spectra were also characteristic of the liquid crystalline spectra from fatty acid

Figure 1.2

^2H NMR spectra of galactosyl ceramide at 30°C and 50°C in unsonicated bilayers of DMPC. Spectra are shown from top to bottom for N-stearoyl [d_{35}] GalCer (the "non-OH isomer"), and the L and D isomers of N-[α-OH]stearoyl [d_{34}] GalCer respectively. Corresponding depaked spectra are displayed to the right of each powder spectrum. For the 30° non-OH spectra arrows indicate spectral features associated with the plateau region referred to in the text. In each case, the glycolipid comprised 10 mol % of total membrane lipid.

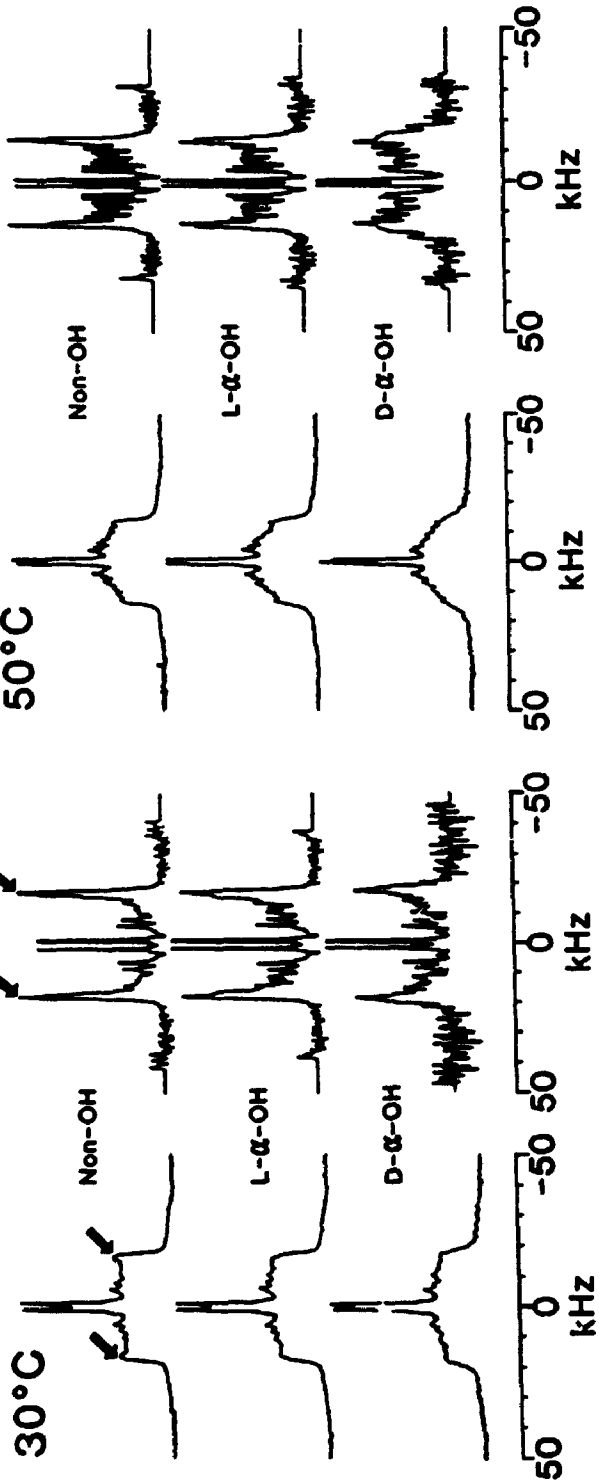

30°C
50°C
Non-OH
L-α-OH
D-α-OH
50
0
-50
kHz

TABLE 1.1

^2H NMR spectral data corresponding to Figure 1.2 for N-stearoyl[d_{35}] galactosyl ceramide, and D- and L-α-hydroxy N-stearoyl[d_{34}] galactosyl ceramide isomers respectively in fluid bilayers of dimyristoyl phosphatidylcholine. Data was listed for 30°C and 50°C. Glycolipid comprised 10 mol% of membrane lipid. $\Delta\nu_Q$ refers to measured spectral quadrupolar splittings; S_{CD} is the calculated order parameter. "Carbon Number" refers to the location of the spectral probe on the glycolipid fatty acid chain (carboxyl carbon as C_1). Peak assignments are based on peak intensities relative to the integrated total, assuming a gradient of decreasing motional order to C_{18}. "Plateau Region" refers to unresolved resonances giving rise to the intense outermost spectral peaks. The plateau region covers C_3 - C_{10} at 30°C and C_3-C_9 at 50°C. The peak width at half height of this spectral feature is listed as a measure of the spread in values of quadrupolar splitting for methylene groups in the plateau region.

Table 1.1: Quadrupolar splittings for deuterated glycosphingolipids in DMPC at 30°C and 50°C.

		Non-OH		D-α-OH		L-α-OH	
Carbon Number		$\Delta\nu_Q$ (kHz)*	S_{CD}	$\Delta\nu_Q$ (kHz)*	S_{CD}	$\Delta\nu_Q$ (kHz)*	S_{CD}
30°C	C_{18}	2.9	0.022	2.4	0.019	2.6	0.020
	C_{17}	12.5	0.098	11.8	0.093	12.7	0.100
	C_{16}	17.0	0.133	15.8	0.124	17.2	0.135
	C_{15}	22.6	0.177	20.5	1.161	21.8	0.171
Plateau region Centre		35.2	0.276	35.6	0.279	34.2	0.268
Plateau region Width(kHz)		2.2		3.1		3.2	
50°C	C_{18}	2.4	0.019	1.6	0.013	1.9	0.015
	C_{17}	8.4	0.066	8.4	0.066	8.7	0.068
	C_{16}	10.8	0.085	10.2	0.080	11.0	0.086
	C_{15}	13.6	0.107	14.0	0.110	14.5	0.114
	C_{14}	15.8	0.124	15.3	0.120	16.1	0.126
	C_{13}	18.6	0.146	19.3	0.151	19.5	0.153
Plateau region Centre		28.2	0.221	29.7	0.233	28.0	0.220
Plateau region Width(kHz)		2.0		4.1		2.7	

* Values obtained from depaked spectra. Estimated error ± 0.5 kHz.

perdeuterated GalCer in this present study. This was in accord with previous studies of pure N-palmitoyl GalCer (Skarjune and Oldfield, 1979). Specifically, if one examined the spectra for the various GalCer species, a buildup of intensity, or shoulder, at the outer edges of the spectra was evident (Fig. 1.2). This was a result of overlapping peak intensity for positions C-3 to approximately C-10 of the fatty acid chain for all three species. This "plateau region" was indicative of very similar orientational order in this region of the fatty acid chain. The assignments of the resolved peaks in the depaked spectra were based on literature data derived from pure phospholipids and GalCer deuterated in the fatty acid (Smith and Mantsch, 1982; Seelig, 1977; Skarjune and Oldfield, 1979; Oldfield et al., 1978; Davis, 1979). In general, for saturated species, the innermost peaks correspond to the terminal end of the fatty acid, C-18 following sequentially outward to C-17, C-16 etc. The progression reflected the increasing order of carbons in the fatty acid chain towards the outer surface of the membrane bilayer. As can be seen in Fig. 1.2, the resolution of the peaks at higher temperature allowed more extensive assignments (see Table 1.1). Using equation (2), the plateau region order parameters (S_{CD}) for the various GalCer species was calculated (Table 1.1). These values were slightly higher than those observed for DMPC (Oldfield et al., 1978) or DPPC (Davis, 1979) which ranged from 0.22 to 0.25, in the liquid crystalline phase. This was consistent

with previous observations of higher order being displayed by the fatty acid of GSL's in a given host matrix as compared to the phospholipid fatty acids (Grant, 1987, Sharom et al., 1976).

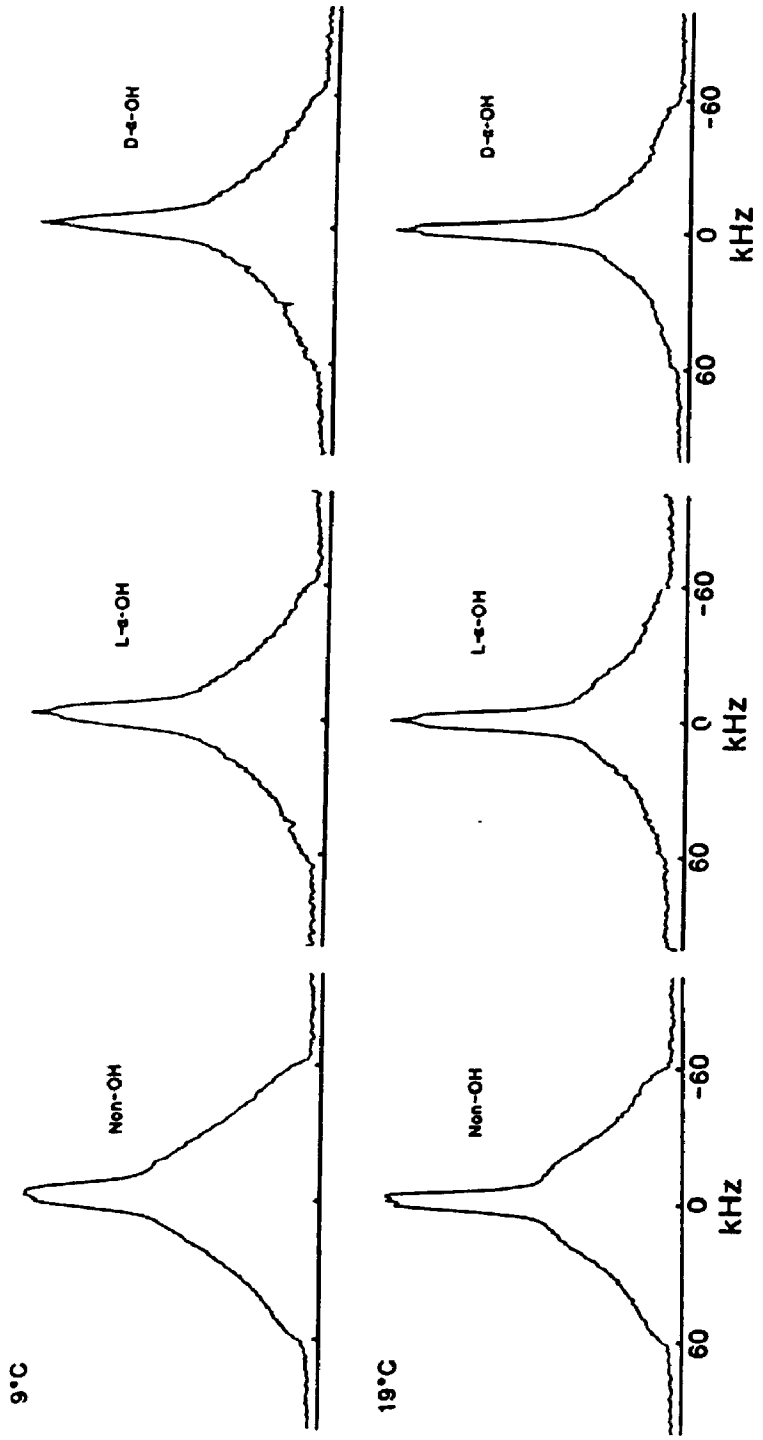
The spectra at both 9°C (below the 15°C pretransition of DMPC) and 19°C (between pretransition and the gel-to-liquid crystalline transition temperature of 23°C for DMPC) all showed characteristic gel phase features. They were very similar to the spectral features from spectra of DPPC with perdeuterated fatty acid chains in the gel phase (Davis, 1979).

1.3.2 *Contrasting α-hydroxy with the non-hydroxy analogue in phosphatidylcholine bilayers*

The most noticeable difference in the spectra between the hydroxy and non-hydroxy GalCer species occurred in the plateau regions. The non-hydroxy sample displayed sharp shoulders in the powder spectra (Fig 1.2) indicating the very similar ordering of the methyenes C-3 to approx C-10 at both 30°C and 50°C. This was in contrast to the hydroxy derivatives which show varied degrees of rounding in their shoulder regions. The rounding of the shoulders in the spectra was more striking at higher temperatures which was made more evident in the depaked spectra. This indicated that the plateau region approx. C-3 to C-10 of the hydroxy derivatives displayed non-uniform orientational order.

Figure 1.3

Gel phase ^2H NMR spectra of galactosyl ceramide at 9° C and 19° C in unsonicated bilayers of DMPC. From left to right: N-stearoyl [d_{35}] GalCer, D-α-OH [d_{34}] GalCer, and L-α-OH [d_{34}] GalCer. In each case, the glycolipid comprised 10 mol% of the membrane lipid.


9°C
19°C
Non-OH
L-α-OH
D-α-OH
60
0
-60
kHz

The non-uniform orientational order was consistent with the α-hydroxy group of the acyl chain acting to disrupt acyl chain packing in the plateau region near the bilayer surface. This effect seemed to be greater for the naturally occurring D-isomer.

Upon close examination of S_{CD} for the various species recorded in Table 1.1, a slight decrease in fatty acid chain order towards the methyl terminus of the naturally occurring D-α-OH isomer was noticed as compared to the non-hydroxy isomer. The difference was close to experimental error. The values for the plateau region, measured from the middle of the feature, were similar between the non-hydroxy and L-α-OH species. The value for the naturally occurring D-isomer was larger at 30°C than the L isomer and at 50°C it was clearly larger than both L-α-OH and the non-hydroxy species.

Upon comparison of the gel phase spectra at 9°C and 19°C (Fig. 1.3), some differences and similarities between spectra were seen. The gel phase spectra of the L- and D-α-OH GalCer derivatives were virtually identical to each other but were both different from that of the non-hydroxy GalCer species. The non-hydroxy spectrum at 9°C covered approximately ±63 kHz while the hydroxylated species' spectrum covered approximately ±60 kHz. This suggested that the non-hydroxy glycolipid rotation rate was slow or that there was a decrease in the gauche population as compared

to the hydroxy derivative. The large increase in intensity in the centre of the spectra for the hydroxy derivatives likely indicated increased chain motion or disorder as compared to the non-hydroxy species. The spectra at 19° C are consistent with the aforementioned characteristics at 9° C.

1.4 CONCLUSIONS

The fatty acid hydroxyl function has been speculated to be involved in hydrogen bonding thereby controlling GSL conformation (Pascher, 1976,Pascher and Sundell, 1977; Pearson and Pascher, 1979; Nyholm et al., 1990) and possibly in GSL-GSL attraction in fluid DMPC (Neuringer et al., 1979). It has also been considered to contribute to the increased order seen in membranes when GSLs are added (Curatolo, 1987, 1987a). In addition, there is the potential for steric effects of the hydroxyl function which could serve to disrupt acyl chain packing.

In the experiments described herein, GSLs were studied at 10 mol% in an effort to approach biologically relevant systems. At this concentration, the GSLs studied were dispersed in the fluid DMPC matrix at 30° C and 50° C. The results mimicked the findings in a similar system with glucosylceramide at 17 mol% in DMPC. At 9° and 19° C the GSLs were likely somewhat phase separated in the DMPC gel phase (Skarjune and Oldfield, 1982; Ruocco et al., 1983; Mehlhorn et al., 1989). In fluid membranes, it appears that the

fatty acid hydroxyl function of GalCer led to the disruption in the uniformity of the plateau region (approx. C-3 to C-10). The naturally occurring (D) isomer displayed a higher degree of order in the plateau region when compared to either the (L) isomer or the non-hydroxy species. Generally though, all 3 species examined in the liquid crystalline phase exhibited very similar behaviours. The hydroxyl function, (D) or (L), led to a decrease in order in the gel phase as compared to the non-hydroxy GSL. It would appear that the hydroxyl function, replacing hydrogen, may have greater local area requirements in phospholipid membranes thereby serving to disrupt acyl chain packing. This was consistent with studies involving the hydroxylated fraction of naturally occurring GalCer which found a lowering of the temperature and enthalpy of the phase transition and reduced order in the gel phase (Bunow and Levin, 1980; Curatolo and Jungalwala, 1985; Maggio et al., 1985; Bunow, 1979).

CHAPTER 2

THE INFLUENCE OF AN UNSATURATED FATTY ACID ON THE BEHAVIOUR OF A GLYCOSPHINGOLIPID IN PHOSPHATIDYLCHOLINE BILAYERS

2.1 INTRODUCTION

GSL's are carbohydrate bearing lipids which are thought to function as structural elements in eucaryotic plasma membranes and also to be involved in a number of other roles (Curatolo, 1987a; 1987b). Their arrangement and behaviour are important factors that govern these functions (Curatolo, 1987a; 1987b; Grant, 1987; Thompson and Tillack, 1985). Thus, the investigation of GSL arrangement and behaviour in membrane environments is essential for insight into the processes in which GSLs participate.

The N-acyl fatty acid, which is part of their ceramide backbone (Fig. 2.1), has been widely claimed to be an important factor in the modulation of GSL behaviour. While the occurrence of an unsaturated fatty acid on carbohydrate bearing lipids is common, very few studies have dealt with them.

The effect of unsaturation on behaviour of the pure hydrated

naturally occurring species, N-(oleoyl)-GalCer, has been measured for comparison to the saturated analogue, N-(stearoyl)GalCer (Reed and Shipley, 1989). N-(stearoyl) GalCer has a gel to liquid phase transition of 85°C. The unsaturated derivative, N-(oleoyl)-GalCer, displayed more complex behaviour as studied with differential scanning calorimetry. It shows a transition from gel to a metastable phase upon warming to 44.8°C. After a period of approximately 24hr, a new gel phase with a phase transition of 55.5°C was observed. Thus one effect of the unsaturation, on the fatty acid of GalCer, is the decrease in the main transition temperature. This effect has been observed in phospholipids but the magnitude of the effect is much greater than it is with the GSL, GalCer. 1-palmitoyl-2-stearoyl-phosphatidylcholine has a main transition temperature of 49°C while that of the 2-oleoyl (POPC) derivative is -3°C (Curatolo, 1986, Coolbear et al., 1983).

We (Singh et al., 1992) were the first to address the effect of GSL fatty acid unsaturation in mixed membranes. We used ^{2}H-NMR to study the behaviour of N-(oleoyl)-GalCer at low concentrations in phosphatidylcholine bilayers. Glycerolipid systems, possessing deuterated oleic acid, have been studied previously (Seelig and Waespe-Sarcevic; 1978,Hinz and Sturtevant, 1972; Seelig and Seelig, 1977). The data from these systems was used in the present investigation for comparison and analysis.

2.2 **MATERIALS AND METHODS**

2.2.1 *Source of materials*

1,2-dimyristoyl and 1-palmitoyl-2-oleoyl phosphatidylcholines (DMPC and POPC respectively) were obtained from Avanti Polar Lipids, Birmingham, AL, and were used without further purification. Galactosyl ceramide (GalCer) (Type II from beef brain) was obtained from the same commercial source. Lyso-GalCer was produced from the above material by hydrolysis in refluxing butanolic KOH (Taketomi and Yamakawa, 1963) and was ninhydrin positive. Lyso-GalCer was applied to a column of Bio Rad silicic acid 200-400 mesh and eluted with a gradient of methanol in chloroform. Perdeuterated oleic acid (Cambridge Isotope Laboratories) was attached to lyso-GalCer via a coupling procedure using dicyclohexylcarbodiimide to produce N-oleoyl-[d_{33}]-GalCer (Sharom and Grant, 1975). Reactions and purification were followed by thin layer chromatography on Merck silica gel 60 plates, eluted with 65:15 $CHCl_3/CH_3OH$, and developed with ninhydrin or sulphuric acid/ethanol spray. Organic solvents were reagent or spectral grade.

2.2.2 *Sample preparation*

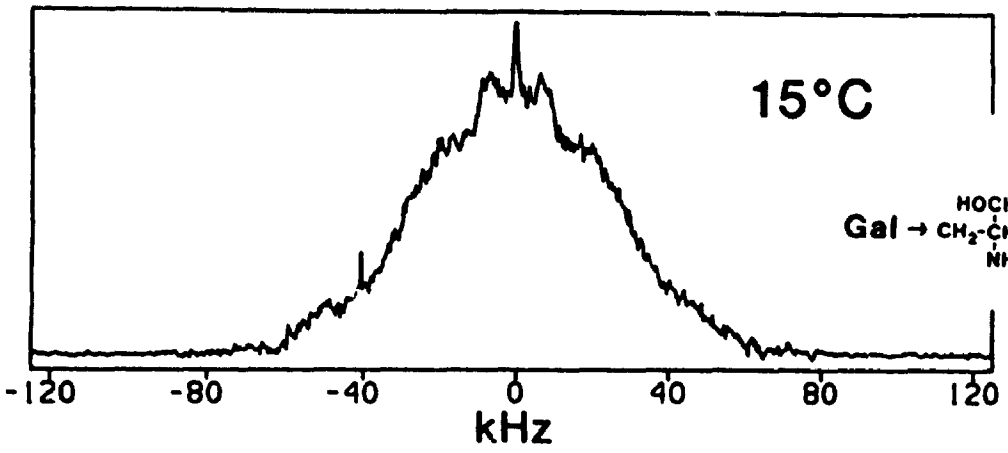
Lipid samples were prepared for spectroscopy by dissolving all components at the desired final ratio in 2:1 (by volume) $CHCl_3/CH_3OH$ and

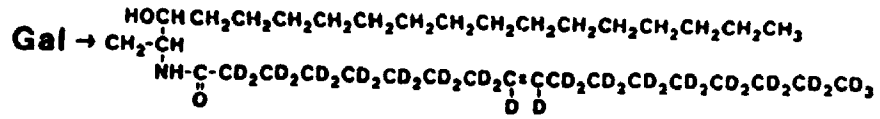
removing solvent by evaporation under a stream of nitrogen. They were then maintained under vacuum (rotary pump) for at least 3 h to remove traces of solvent. The resultant lipid films were successively hydrated with 150 μl of deuterium depleted water (MSD Isotopes) and lyophilized three times, prior to final rehydration with deuterium depleted water (total volume 150-200 μl). They were then subjected to 8 freeze-thaw cycles during which the temperature was raised above the main transition of the lipid matrix involved and then the sample was vortexed.

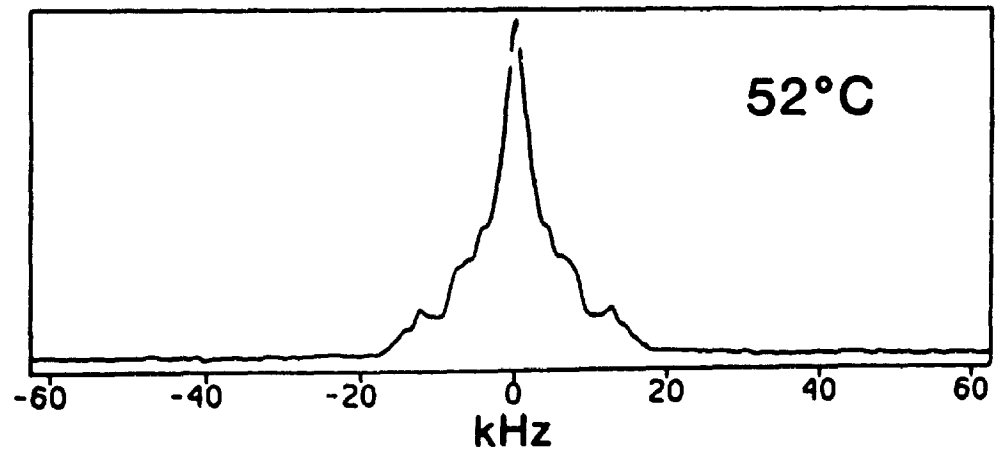
2.2.3 *Acquisition of spectra*

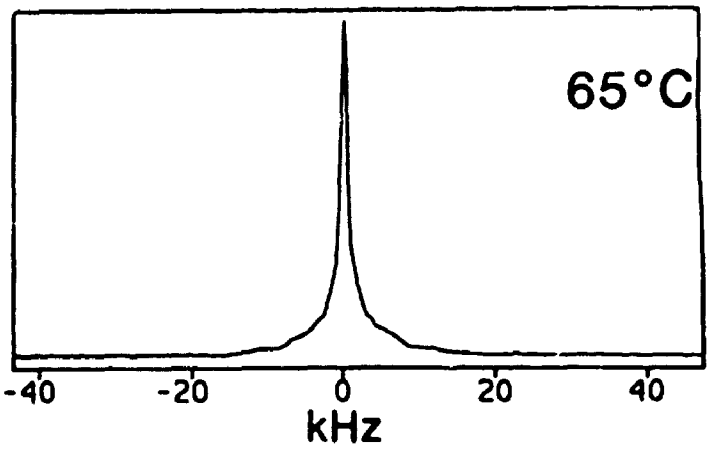
^{2}H NMR spectra in Figure 2.3 (excluding 2.3A) were acquired at 30.7 MHz on a "home-built" spectrometer (Singh et al., 1992). Spectra were recorded using a quadrupolar pulse sequence (Davis et al., 1976) with full phase cycling and quadrature detection (Perly et al., 1985). All other ^{2}H NMR spectra were acquired at 55.26 MHz on a "home-built" spectrometer (Singh et al., 1992a), and were recorded using the quadrupolar echo sequence (Davis et al., 1976) with full phase cycling and quadrature detection (Davis, 1983). The 90° pulse lengths were 1.9 μs and the separation between the two pulses in the quadrupolar echo was 30 μs. The time domain quadrupolar echo signal was filtered and symmetrized about the top of the echo using a procedure described elsewhere (Davis, 1983; Vist and Davis,

Figure 2.1: ^2H NMR spectra of pure hydrated N-oleoyl [d_{33}] galactosyl ceramide (GalCer) at 15°C, 52°C, and 65°C. The chemical structure of the probe labelled glycolipid is shown as an insert. The sample comprised 5 mg (5.8 μmol) of deuterated GSL and was run at 15°C, prior to warming to the higher temperatures with an equilibration time of 30 min.


15°C
-120
-80
-40
0
40
80
120
kHz


Gal → CH2-CH
HOCH CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH3
NH-C-CD2CD2CD2CD2CD2CD2CD2C=CCD2CD2CD2CD2CD2CD2CD2CD2CD3
O
D D


52°C
-60
-40
-20
0
20
40
60
kHz


65°C
-40
-20
0
20
40
kHz

1990). Spectral simulations and moment analyses were performed on a μVAX II computer interfaced to a "home-built" data acquisition computer (Singh et al., 1992).

2.3 **RESULTS AND DISCUSSION**

2.3.1 *In General*

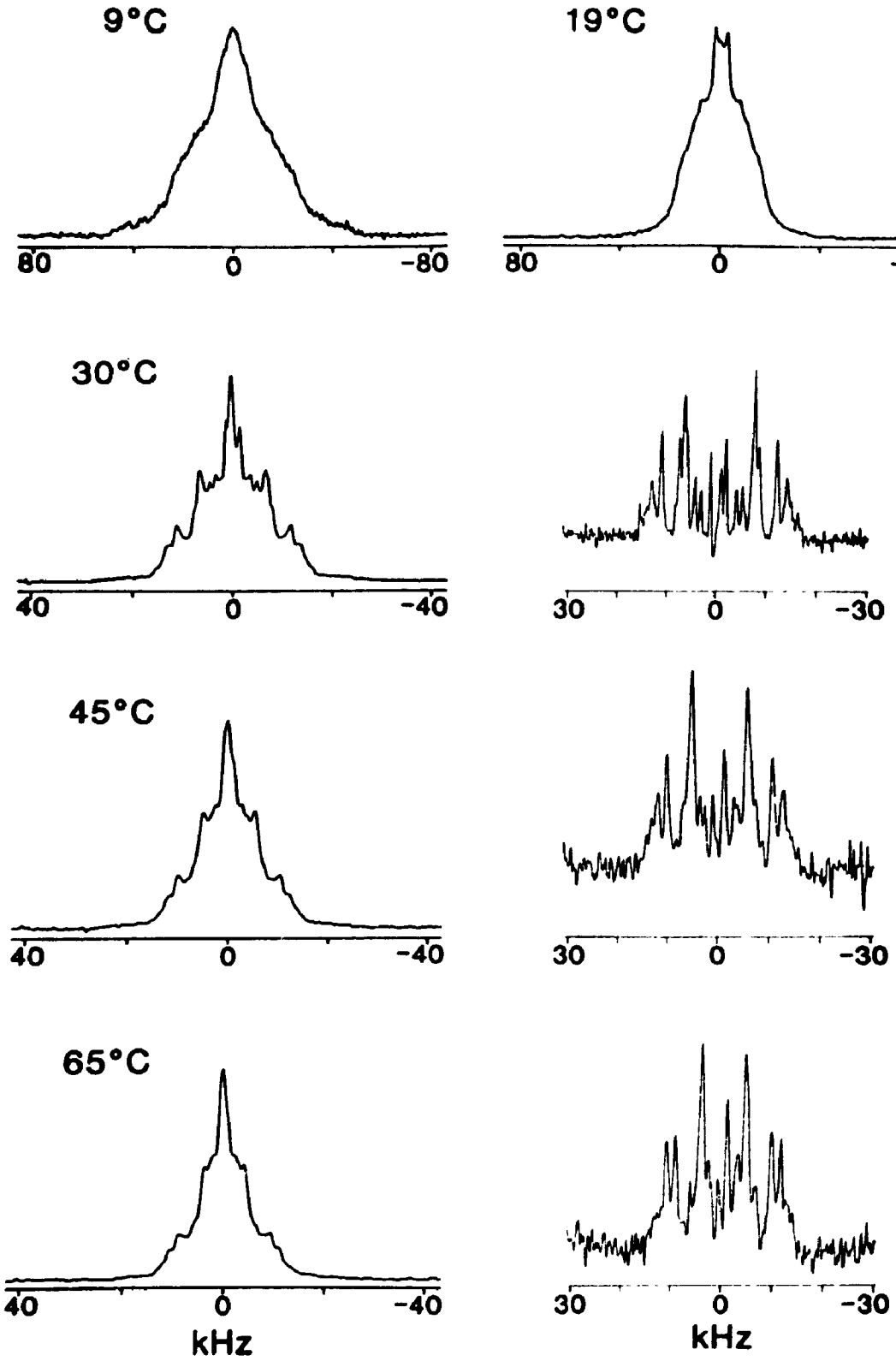
The carbohydrate headgroup of the GSL is quite different from the zwitterionic phosphate headgroup of phosphatidylcholine. The existence of potential GSL hydrogen bonding groups has been speculated to differentiate the behaviour of GSLs from phospholipids (Curatolo, 1987a; 1987b). Nevertheless, the *sn-2* fatty acid in phosphatidylcholine (Pearson and Pascher, 1979) when compared to the fatty acid of GalCer (Pascher and Sundell, 1977) has been shown, using X-ray crystallography, to be in the same conformation.

At 15°C, the spectrum of pure perdeuterated N-(oleoyl)-GalCer displayed characteristic gel phase features (fig. 2.1) indicative of very slow motion. Upon warming to 52°C, the liquid crystalline spectrum obtained within 5hr of warming suggested that the transition from gel to liquid crystalline states has occurred. In the liquid crystalline state, the GSL acyl chain undergoes much greater motion as compared to the gel phase at 15°C. The central unsplit peak likely resulted from the overlap of signals from

highly curved liposomes and HOD. The highly curved liposome permits rapid reorientation of the GalCer molecules via lateral diffusion and the rapid tumbling of the whole liposome itself. This is consistent with the fact that pure GalCer forms liposomes with a size range of 100 to 500nm (Curatolo and Neuringer, 1986,Maggio et al., 1988). At 65°C, all of the quadrupolar couplings, seen at 52°C, collapsed into a single intense peak. This likely indicated a reduction in size of the average liposome in the sample. Overall, the spectra in Fig. 2.1 are consistent with studies (using DSC and X-ray diffraction) of pure N-(oleoyl)-GalCer. In the temperature range that these recent studies encompassed, the lipid structures generated were reported to be in bilayer form.

To more closely approximate a biological setting, N-(oleoyl)-GalCer was studied in a phosphatidylcholine matrix at 10 mol%, since GSLs are usually present as minor components in cellular membranes. The spectra of N-(oleoyl)-GalCer at 10 mol% in dimyristoyl phosphatidylcholine (DMPC), are presented in Fig.2.2. At 9°C, the spectrum was characteristic of gel phase lipid. However, at 19°C, the spectrum still possessed gel phase features but the narrowing of features gave evidence of some glycolipid motion. The spectra taken at 30°C, 45°C and 65°C did not show any evidence of gel phase and were indicative of a liquid crystalline phase. At 30°C, the lipid sample was below the pure N-(oleoyl)-GalCer liquid

Figure 2.2: ^2H NMR spectra of N-oleoyl [d_{33}] GalCer incorporated at 10 mol% into fully hydrated bilayers of the phospholipid, dimyristoyl phosphatidylcholine (DMPC) at selected temperatures. Depaked spectra are shown to the right for samples run at 30°C, 45°C, and 65°C. Each sample contained 5 mg (5.8 μmol) of deuterated GSL.


9°C
80
0
-80
19°C
80
0
-80
30°C
40
0
-40
30
0
-30
45°C
40
0
-40
30
0
-30
65°C
40
0
-40
kHz
30
0
-30
kHz

crystalline transition temperature of 44.8°C (Reed and Shipley, 1989). The liquid crystal spectrum argues that the unsaturated GalCer was, for the most part, completely dispersed in the DMPC host matrix despite C-18:1 GalCer's high transition temperature as compared to that of DMPC (23°C). This was consistent with previous studies of GalCer (Mehlhorn et al., 1989; Fenske et al., 1991). This was also seen in experiments with glucosylceramide at 17% in DMPC (Skarjune and Oldfield, 1982). It should be noted that if there was a very small amount of phase separation in the samples at 30°C, it may not have been detectable since the liquid crystalline features would have "masked" gel features resulting from the presence of a very small amount of gel phase lipid.

2.3.2 *Contrasting deuterated N-(oleoyl)-GalCer with glycerolipids - not too much contrast!*

The assignment of the spectral features from the spectra of deuterated N-(oleoyl)-GalCer has not been done in past. We are the first to have attempted this. Experiments involving deuterated oleic acid species introduced to living systems, specifically *Escherichia coli* (Gally et al., 1979) and *Acholeplasma laidlawii* (Rance et al., 1980), have been done. In addition, deuterated oleic acid in POPC has also been studied (Seelig and Waespe-Sarcevic, 1978,Seelig and Seelig, 1977). Despite the fact that the

systems, in which the deuterated oleic acid was incorporated, are very different (two species of organisms and an artificial pure POPC system) the results from each system were very similar. The only major difference was at the C_2 methylene position which is close to the membrane surface. This is remarkable when noting the fact that the living systems have a plethora of diversity in their membranes, all of which would contribute to the membrane's character. This variety is in stark contrast to the simplicity of the pure phospholipid system of POPC.

Noting the similarity between the published data for deuterated oleic acid incorporated in the above glycerolipid systems in *sn-2* linkage, a computer simulated spectrum was generated. The data from published experiments with POPC (Seelig and Waespe-Sarcevic, 1978; Seelig and Seelig, 1977) supplemented with data from *A. laidlawii*, along with some interpolated points, (see Table 2.1) were used to do this (Fig. 2.3). The peak assignments of the simulated spectrum were based on the published data used to generate it. The spectrum of perdeuterated N-(oleoyl)-GalCer at 30°C in DMPC at 10 mol% was compared to this and was observed to be virtually identical (Fig. 2.3). From this identity, the assignment of the spectrum from perdeuterated N-(oleoyl)-GalCer in DMPC was performed. In comparing the 30°C, 45°C and 65°C spectra after assigning the peaks in the "depaked" or 90° orientation simulation, it was observed that the fatty

Figure 2.3: Comparison of ^2H NMR spectra for deuterated oleic acid attached to a substituted glycerol vs a glycosyl sphingosine backbone, in fluid bilayer membranes. A) Computer simulated spectrum for [d_{33}] oleic acid attached in *sn*-2 linkage to glycerolipids (from literature data for 1-palmitoyl-2-oleoyl phosphatidylcholine at 27°C (Seelig, J. and Waespe-Sarcevic, N. 1978; Seelig and Seelig, 1977), and *Acholeplasma laidlawii* membranes at 25°C (Rance et al., 1980) - see caption to Table 2.1 and Figure 2.4). B) Experimentally derived spectra for N-oleoyl [d_{33}] GalCer at 10 mol% in fluid DMPC at 30°C. C) Experimentally derived spectra for N-stearoyl [d_{35}] GalCer at 10 mol% in fluid DMPC at 30°C. D) Experimentally derived spectra for N-oleoyl [d_{33}] GalCer at 5 mol% in fluid POPC at 30°C. In each case the powder spectrum is shown on the left, and the depaked spectrum on the right. Arrows in 2.3A indicate accepted spectral assignments from the literature quoted, as listed in Table 2.1. The single arrow in 2.3B,D indicates the position of the sharp, unsplit central peak which is not expected to be present in the simulated spectrum.

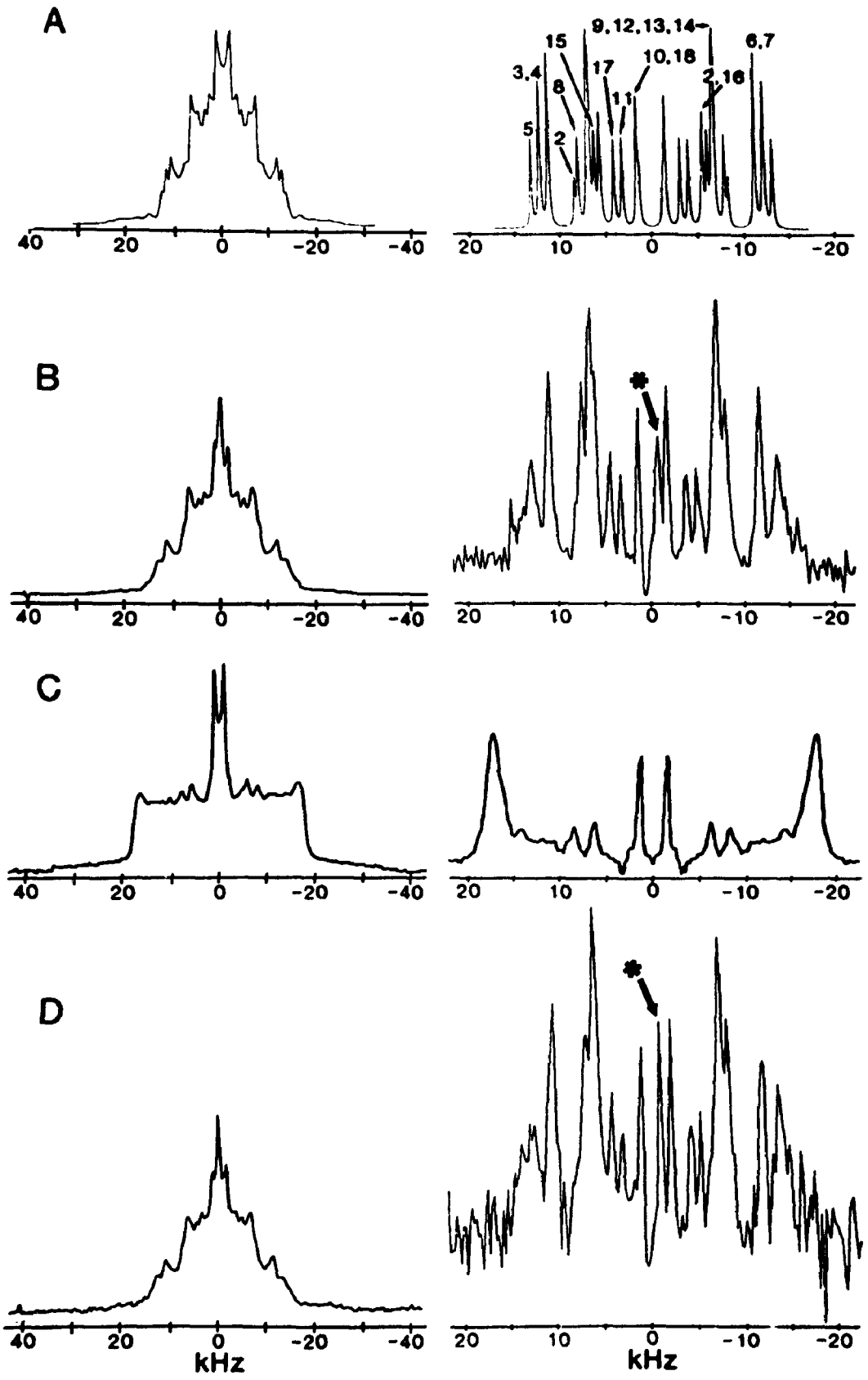

A
9,12,13,14
15
6,7
10,18
17
3,4
8
2,16
11
5
2
40
20
0
-20
-40
20
10
0
-10
-20
B
*
C
D
*
kHz
kHz

Figure 2.4: Plot of the order parameters, S_{CD}, for deuterated oleic acid attached in *sn*-2 linkage to glycerolipids, taken from the literature (Seelig, J. and Waespe-Sarcevic, N. 1978; Seelig and Seelig, 1977), Rance et al., 1980) used to produce Figure 2.3A above (■), superimposed upon tentative assignments based on correspondence for N-oleoyl [d_{33}] GalCer at 10 mol% in fluid DMPC at 30°C (+). Values for carbons #13 and 15 in the glycerolipid plot were interpolated by drawing a smooth curve through points on either side.

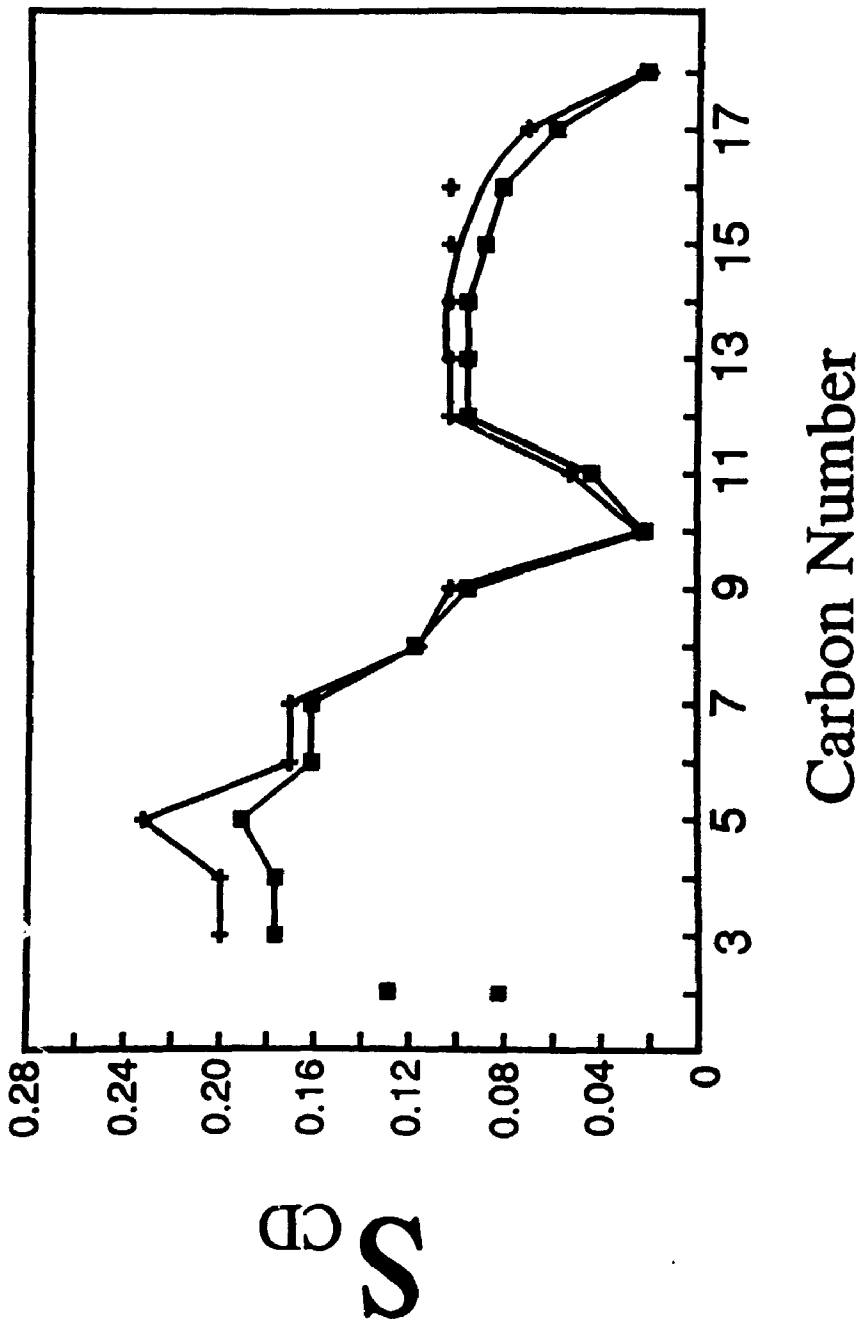

S_{CD}
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
3
5
7
9
11
13
15
17
Carbon Number

Table 2.1: Literature ^2H NMR spectral data for deuterated oleic acid attached at the *sn*-2 position of glycerol based lipids in fluid membranes, tabulated for comparison with experimental data from samples of N-oleoyl [d_{33}] GalCer in various fluid phosphatidylcholine bilayers. Quadrupole splittings for pure POPC, and for *Acholeplasma laidlawii* were taken from references Seelig, J. and Waespe-Sarcevic, N. 1978; Seelig and Seelig, 1977 and Rance et al., 1980 respectively. Literature data are presented as a function of ^2H location along the fatty acid chain ("Carbon #") with the corresponding value of $\Delta\nu_Q$ (values for carbons # 13 and 15 were interpolated by drawing a smooth curve through *Acholeplasma* data points (Rance et al., 1980). Experimental data are presented as a function of resolvable quadrupolar splitting, $\Delta\nu_Q$ from high to low, with the corresponding suggested ^2H location(s) assigned by comparison with the simulated spectrum, Figure 2.3A.

Literature $\Delta\nu_Q$ Values (kHz) for ^2H in the Oleate Chain

Carbon No.	A. laidlawii (25°C)	POPC (27°C)
2	20.0 25.5	10.8 16.4
3	23.8	
4	24.0	
5	25.8	
6	22.0	
7	22.0	
8	15.6	15.6
9	14.8	13.4
10	4.3	2.5
11	7.8	6.2
12	13.5	13.0
13	13.3	
14	13.0	
15	12.0	
16	11.0	
17	8.0	
18	3.0	

Experimental $\Delta\nu_Q$ Values (kHz) for ^2H in the Oleate Chain on GalCer

Carbon No.	DMPC (30°C)	POPC (27°C)	DMPC (65°)
5	31.6	28.8	27.0(?)
3&4	27.2	26.4	22.6
6&7	23.2	22.6	18.8
8&2(?)	15.8	15.0	12.7
2(?),9,12, 12,13,14, 15,16	14.0	13.2	8.7
17	9.6	9.4	
11	7.2	7.4	
18&10	3.2	3.0	1.8

acyl chain became more disordered as the temperature is increased (Table 2.1). Some shifting of the relative peak positions is also noted.

2.3.3 *Comparing unsaturated GalCer with saturated GalCer*

The spectra of N-(stearoyl)-GalCer and N-(oleoyl)-GalCer, both in DMPC at 30°C, are shown in Fig. 2.3. The relative shapes of the two spectra were very different. This was a reflection of the very different orientations of the methylene groups in the region of C-8 to C-11. The *cis* 9,10 double bond has been found to make an angle of 7-8° to the bilayer normal (motional director) in studies with deuterated POPC (Seelig and Seelig, 1977). This results in reduced quadrupolar splittings for the deuterons at methylenes C-9 and C-10. The effect of the *cis* double bond also extended to the methylene carbons above and below this region. Fig. 2.4 is a plot of S_{CD} (order parameter) for perdeuterated N-(oleoyl)-GalCer in DMPC. S_{CD} was calculated from literature data used to generate the previously mentioned simulated spectrum. The shape of this S_{CD} profile as a function of position in the fatty acid chain was very different from the classic pattern seen for the saturated fatty acid analogue (Seelig, 1977; Smith, 1984; Davis, 1983). The effect of the *cis* double bond was evident for the acyl chain region C-8 to C-11 which manifests much smaller quadrupolar splittings. This has been demonstrated to arise from the fact

that the double bond makes an angle of 7-8 degrees to the bilayer normal (Seelig and Seelig, 1977). The double bond also exerts a measurable effect on the methylene carbons above and below it in the chain resulting in lowered S_{CD} values from those seen for saturated analogues. The striking similarity between the GalCer system and the glycerolipid systems was notable.

2.3.4 *Unsaturated GalCer in an unsaturated matrix*

The systems, from which the assigned deuterated oleic acid data were obtained (Seelig and Waespe-Sarcevic, 1978,Seelig and Seelig, 1977,Rance et al., 1980), dealt with membranes which are composed mostly of lipids possessing unsaturated fatty acids. To more closely resemble these systems, deuterated N-(oleoyl)-GalCer was incorporated, at 5 mol%, in POPC bilayers. This sample was run at 27°C which is well above the fluidus shown in the literature POPC/GalCer phase diagram (Curatolo, 1986). The spectrum resulting from this sample was virtually identical to that of the glycolipid in DMPC and the simulated spectrum (see Fig. 2.3). Thus the effect of the unsaturated host matrix was similar to saturated (DMPC) in the control of C-18:1 GalCer lipid properties as monitored from its deuterated fatty acid.

2.4 **CONCLUSIONS**

Perdeuterated N-(oleoyl)-GalCer was studied in pure hydrated form, in DMPC, a saturated phosphatidylcholine matrix and in POPC, a *sn-2* unsaturated host matrix. Several different temperatures were considered. This was in an effort to gain insight into the effect of an unsaturated fatty acid on the phase behaviour and dynamics of GalCer, since the existing literature dealt only with glycerolipid systems.

In general, the effect of an unsaturated fatty acid on the behaviour of GalCer in phosphatidylcholine bilayers was virtually indistinguishable from that of the glycerolipid systems of *A. laidlawii* and pure POPC. The prominent effect of the *cis*-9,10 double bond on the ^{2}H-NMR spectrum of the GSL fatty acid was very similar to that seen for the *A. laidlawii* and pure POPC systems. The observed effect that the *cis* unsaturation had on the ^{2}H-NMR spectrum of the unsaturated fatty acid residue stems from the double bond making an angle of approximately 7-8° with the bilayer normal (Seelig and Seelig, 1977). It was striking that unsaturated fatty acid behaviour of the model membrane binary systems of this study so closely resembled the observations made with the *A. laidlawii* and the pure POPC systems.

CHAPTER 3

GLYCOSPHINGOLIPID PHASE BEHAVIOUR AND ACYL CHAIN ORDER

3.1 INTRODUCTION

Properties, such as physical arrangement and motional characteristics are thought to modulate the behaviour of GSLs in their role as recognition sites, in addition to controlling the structural effects that they have on membranes (Curatolo, 1987a, 1987b; Thompson and Tillack, 1985; Grant, 1985). Studies in this field have begun to lay a foundation for the understanding of the forces involved in these GSL roles. Many such studies have been done using pure GSL systems, a simplicity which allows detailed interpretation (Curatolo, 1987a; Maggio et al., 1981). To further understand the forces at work in a membrane context, these studies must be extended to include more complicated systems, such as GSL mixtures with phospholipids. One approach to a binary system, with an interest in molecular arrangement and dynamics, would be the study of phase behaviour. Another approach, on a more detailed level, could involve the investigation of the behaviour of a specific molecular region of interest (eg. the fatty acid chain of the GSL).

The effects of GSL fatty acid length on GSL behaviour has been

examined in classic literature studies of receptor cryptic ity. It was noted that GalCer, with a long chain fatty acid, was a better receptor for antibodies than the shorter chain analogue in a DSPC/cholesterol host matrix at 23°C (Alving et al., 1980; Schichijo and Alving, 1985). A similar observation has also been reported with systems containing sulphatide (Crook et al., 1986). To account for these observations, it has been suggested that the headgroup of GalCer with a long chain fatty acid, may protrude further above the membrane surface than the shorter chain analogue. Alternatively, it has been suggested that the lateral distribution of the GSL in the membrane may be the key to the difference in reactivity. This hypothesis stems from the fact that factors affecting immune agglutination of liposomes, such as fatty acid chain length of the host matrix, cholesterol content and temperature, are known to be able to alter lipid lateral distribution (Utsumi et al., 1984).

The phase behaviour of a number of GSLs has been considered via DSC. Experiments with bovine brain gangliosides in SOPC, found that these GSLs were miscible with SOPC when they comprised less than 30% of the system (Bunow and Bunow, 1979). Another study, with a gm_1/egg PC system, found that there was complete miscibility (Bach et al., 1982). The phase behaviour of a number of GSLs has been studied systematically in DPPC bilayers (Maggio et al., 1985a). It was observed that the nature of the GSL

carbohydrate as well as that of the fatty acid were key factors in the GSL phase behaviour. Solid phase immiscibility was indicated at low GSL concentrations (Maggio et al., 1985).

Phospholipids tend to have fatty acids that are 16 to 18 carbons in length while those of GSLs tend to be 18 to 24 or 26 carbons. Pure hydrated N-lignoceroyl GalCer, possessing a 24 carbon fatty acid chain, has a fluid/gel main transition of 82°C. It also shows metastable polymorphism at lower temperatures (Reed and Shipley, 1987). A phase diagram for N-lignoceroyl GalCer in DPPC has been derived using DSC which displayed solid phase immiscibility over the entire composition range studied (Gardam and Silvius, 1989). The phase behaviour of N-palmitoyl GalCer in DPPC has also been studied using DSC. The phase diagram is characterised by solid phase immiscibility and a fluid/gel phase coexistence at GSL mole fractions greater than 0.2, over a substantial temperature range (Ruocco et al., 1983). Phase diagrams for GalCer, retaining its natural fatty acid composition, have been derived for POPC and DPPC host matrices (Curatolo, 1986; Maggio et al., 1985).

The behaviour of the fatty acid tail of a glycolipid species with a fatty acid longer than that of the membrane host matrix is also of considerable interest. More insight would be gained into the forces controlling long chain GSL behaviour. In addition, the problem touches on the question of

communication from one lipid monolayer to the opposing monolayer. The arrangement and dynamics of the terminal portion of a long chain fatty acid of a GSL at low concentration in a short chain host matrix was largely unknown. It had been suggested that the terminal end of these fatty acids "interdigitate" - a phenomenon whereby the omega end of the fatty acid protrudes across the bilayer midplane into the opposing monolayer.

We used ^{2}H-NMR to investigate the phase behaviour of two GalCer/SOPC systems. In one system, GalCer's fatty acid was the same length as the host matrix, while in the other, the fatty acid chain was longer. The fatty acid was either perdeuterated, or selectively deuterated on the omega end. A number of techniques and data manipulations in this study were performed by Dr. M. Morrow and D. Lu - collaborators with our laboratory. Dr. M. Morrow and D Lu ran the spectra and performed mathematical analyses of the data which provided information to construct a phase diagram of C-18 and C-24 GalCer in a SOPC matrix. My role in this study was limited to synthesis, membrane system design and contribution to data analysis. SOPC, a phosphatidylcholine possessing 18 carbon fatty acids, has a main transition temperature of 6°C (Davis and Keough, 1985). This allowed the study of gel phase phenomena without going below the freezing point of water. The formation of ice may interfere with results from the lipid system.

3.2 **MATERIALS AND METHODS**

3.2.1 *Source of materials - synthesis of N-(lignoceroyl-d_{47}) and N-(stearoyl-d_{35}) galactosylceramide*

1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) and galactosyl ceramide (GalCer - from beef brain) were obtained from Avanti Polar Lipids, Birmingham, AL; and were used without further purification. Lyso GalCer (ie. GalCer from which the fatty acid had been removed) was produced from the above material by hydrolysis in refluxing butanolic KOH as has been described previously in chapter 1; and was purified before use on a column of silicic acid (Bic Rad 200-400 mesh), eluting with a gradient of methanol in chloroform. Reactions were followed on Merck silica gel 60 thin layer chromatography plates eluted with 65:15 $CHCl_3/CH_3OH$ and developed with sulphuric acid/ethanol spray. N-lignoceroyl[d_{47}] GalCer was made by converting 31.0 mg (74.5 μmol) of perdeuterated lignoceric acid (Aldrich) to the acid chloride using $SOCl_2$, and subsequently combining this with 32.0 mg (67.1 μmol) of lyso GalCer (Kopaczyk and Radin, 1965). Purification was by silicic acid column chromatography eluting with a chloroform/methanol gradient, as for the lyso compound - yield 55% (31.8 mg (37.0 μmol)) of deuterated GSL. GalCer with 24-carbon fatty acid co-migrated with the

faster-running spot of native beef brain GalCer.

Synthesis, characterisation, and purification of N-stearoyl[d_{35}] GalCer (18:0[d_{35}] GalCer) were *via* similar techniques to those involved with the long chain analogue: 18 mg (56 µmol) of perdeuterated stearic acid (Aldrich) was converted to the acid chloride, and subsequently combined with 27 mg (61 µmol) of lyso GalCer (Kopakczyk & Radin, 1965). Yield was 60% (26 mg (34 µmol)) of deuterated GSL. The 18:0 derivative ran on TLC plates just beneath the C-24 derivative. The specifically deuterated 24-carbon derivative, N-lignoceroyl[d_7] GalCer (24:0[d_7] GalCer), was prepared by coupling lignoceric acid, deuterated on the terminal methyl and the two adjacent methylenes, using the same procedure employed for the perdeuterated analogues.

3.22 *Synthesis of omega d_7 lignoceric acid.*

1,20 eicosanedioic acid dimethyl ester (TCI, Tokyo) was refluxed with $Ba(OH)_2$ in dry methanol, followed by extraction with the addition of HCl / ether (Durham et al., 1967a). After removal of the ether, the resulting monomethyl ester derivative was refluxed with $SOCl_2$ to produce the corresponding acid chloride. The organocadmium product of 1-bromobutane-d_9 (MSD Isotopes) was linked with the above acid chloride (Hubbel & McConnell, 1971) to produce a 24-carbon fatty acid monomethyl

ester with ketone group at C_{20}. The ketone function was reduced by reaction with hydrazine monohydrate (Aldrich) / potassium hydroxide (Durham et al., 1967b). The final product was purified by silicic acid column chromatography eluting with $CHCl_3$. Reactions were followed by thin layer chromatography (Merck Silica Gel 60), eluting with 20/30/1 hexane/ether/formic acid. Identification was confirmed with mass spectrometry and ^1H NMR.

3.23 *Sample preparation and acquisition of spectra*

Mixtures of N-lignoceroyl[d_{47}] GalCer in SOPC were prepared with glycolipid concentrations of 53 mol%, 35 mol%, 24 mol%, 10 mol% and 5 mol%. Because of the limited amount of deuterium-labelled GalCer available, it was necessary to reclaim each sample and use it in preparation of the next. The dry components were co-dissolved in ethanol which was then removed quickly by rotary evaporation at approximately 60°C to prevent preferential precipitation of the less soluble component. The sample was then scraped into an 8 mm NMR tube. The first sample (53 mol%) contained roughly 15 mg of labelled lipid and had a total lipid mass of 23 mg. This sample was hydrated in about 300 µl of 50 mM phosphate buffer at pH 7.0. Following the series of NMR measurements, the hydrated sample was recombined with the residue in the original flask and the water removed

by rotary evaporation with the addition of ethanol. Using the known sample composition, the amount of each lipid in the flask was calculated. The amount of SOPC needed to obtain the next desired composition was determined and added to the flask. The components were then redissolved in ethanol and the process repeated. For all samples, excluding the first, the NMR samples were hydrated in distilled water to avoid increasing the buffer concentrations. The samples with the three highest concentrations each contained approximately 15 mg of labelled lipid. Because of volume limitations, the 10 and 5 mol% NMR samples contained only 5.4 mg and 3.2 mg, respectively, of the labelled lipid.

^{2}H NMR spectra were collected starting from 76°C for the samples containing 53 mol%, 35 mol% and 24 mol% glycolipid, and from 70°C for the remaining samples. The high starting temperature was chosen in order to facilitate equilibrium distribution of components in the bilayer. The small amounts of labelled lipid present required relatively long collection times for each spectrum. In order to reduce the exposure of each sample to high temperatures, spectra were generally collected at 3° intervals. For the lowest two concentrations, spectra were collected at 9° intervals in the highest temperature range (more than 20° from the expected liquidus), 1° intervals in the region of two phase coexistence, and 3° intervals otherwise. GalCer and mixtures containing GalCer are known to exhibit hysteresis in

their thermal behaviour (Reed and Shipley, 1987; Ruocco et al., 1983; Gardam and Silvius, 1989; Reed and Shipley, 1989). Fast cooling of N-lignoceroyl GalCer and its mixtures with DPPC from the liquid crystalline phase has been found to result in metastable gel phases (Reed and Shipley, 1987; Gardam and Silvius; 1989). For mixtures with phosphatidylcholine, fast cooling might also limit lateral redistribution of bilayer components and further prevent the attainment of equilibrium. In the present work, samples were equilibrated above the main transition temperature of each sample for at least an hour. Because of the averaging time necessary to collect each spectrum, subsequent cooling occurred at a rate between 0.5 and 1 °C per hour: this was relatively slow compared to cooling rates employed in the previously mentioned published studies. While it does not totally preclude the possibility of metastable gel phase formation, this cooling protocol was expected to contribute to the maintenance of sample equilibrium as the temperature was lowered.

^{2}H NMR spectra were collected at 23.215 MHz using the quadrupole echo sequence (Davis et al., 1976). The $\pi/2$ pulse length was between 2.1 μs and 2.3 μs. Pulse separation in the echo sequence was 35 μs. The signal-to-noise ratio of the free induction decay was improved by oversampling by a factor of two, applying symmetrization and smoothing to even and odd points separately, and recombining the echoes in the

manner described by Prosser et al (Prosser et al., 1991). Effective digitizer dwell time was thus 4 μs in the liquid crystalline phase and 2 μs for temperatures below the liquidus. For each spectrum, 12,000 to 48,000 transients were collected with a repetition time of 0.6 s. ^{2}H NMR difference spectroscopy (Huschilt et al., 1985; Vist and Davis, 1990; Morrow et al., 1991) was used to determine tie line endpoints in selected two phase regions of the phase diagram for the GalCer (d_{47}) - SOPC mixture.

Fully hydrated lipid bilayer structures containing measured amounts of deuterated C-18 GalCer and selectively deuterated C-24 GalCer in SOPC were prepared as described for the C-24 sample. Samples with the highest deuterium concentrations contained between 15 mg and 17 mg of labelled lipid. 10 mol% and 5 mol% samples contained 5-8 mg because of the limited sample tube volume. ^{2}H NMR spectra for phase diagram generation were collected from 73°C to -14°C, generally at 3° intervals. For the lowest four concentrations, the interval was reduced to 1° in the region of two phase coexistence. Precautions taken to minimise the presence of metastable GalCer phases were as described above (Ruocco et al., 1983; Reed & Shipley, 1987; Gardam & Silvius, 1989; Reed & Shipley, 1989). For the sample having selectively deuterated fatty acid chains, oversampling was by a factor of four so that the effective digitizer dwell times were further doubled.

3.3 RESULTS AND DISCUSSION

The aim of the present study was to examine the phase behaviour of both the long chain (C-24) and the short chain (C-18) GalCer species in SOPC. These were systems in which the GSL possessed a fatty acid chain that was either longer than (C-24) or the same length as (C-18), that of the host matrix. This might be expected to influence molecular arrangement and dynamics. Binary mixtures of phospholipids with fatty acids differing in length have been found to have a tendency to phase separate which is dependent on length. Phospholipid phase separation induced by intramolecular fatty acid mismatch has been observed (Mason, 1988; Gardam and Silvius, 1989). A complication that arises in the interpretation of the results from binary phospholipid mixtures is the alteration of the fluid/gel main transition temperature upon the change of the phospholipid fatty acid. This alteration in phase transition temperature would, by itself, be reason for a difference in phase behaviour of the system. The situation with GSLs is somewhat different since the main transition temperature is primarily determined by the nature of the headgroup (Maggio et al., 1985). In the present study, C-18 and C-24 GalCer, which have about the same transition temperature (82°C), were compared in a SOPC matrix. It thus follows that differences in transition temperatures for the two GalCer species being

compared did not complicate the interpretations from this study. The results bear on the interactions of GSLs with the host matrix and, more specifically, the effect of 6 extra carbons on GSL-phospholipid interactions. As mentioned previously, GSL lateral distribution has been implied as a factor controlling the behaviour of GSLs in membrane environments (Utsumi et al., 1984). As will be seen in chapter 4 and 5, it was found that the different fatty acid chains of GSLs had no major effect on the behaviour of the GSL headgroup but little insight was gained into the lateral distribution of the different GSLs - particularly between the short and long chain GalCer species.

3.3.1 *N-lignoceroyl GalCer spectra*

Fig. 3.1 shows spectra of perdeuterated C-24 GalCer in SOPC in a series of different temperatures and compositions. As a first step in determining the behaviour of C-24 GalCer in the SOPC matrix, several sample conditions were set in order to observe spectral characteristics connected to one particular molecular arrangement. Characteristic liquid crystal spectra were observed for systems above the liquidus (eg.70°C). The concentration of spectral intensity near the centre of the spectra distinguishes them from those well known for perdeuterated phosphatidylcholines (Seelig, 1977; Davis, 1983; Smith,1984) or perdeuterated

Figure 3.1

Concentration dependence of ^2H NMR spectra for N-lignoceroyl[d_{47}] GalCer in SOPC at the temperatures indicated from 1°C to 70°C. Curved arrows in the 1° and 70° spectral groupings indicate features associated with the 24-carbon fatty acid terminal methyl referred to in the text with regard to G2 and liquid crystal phases respectively. Vertical arrows in the 70° spectral group indicate the liquid crystal plateau region edges.

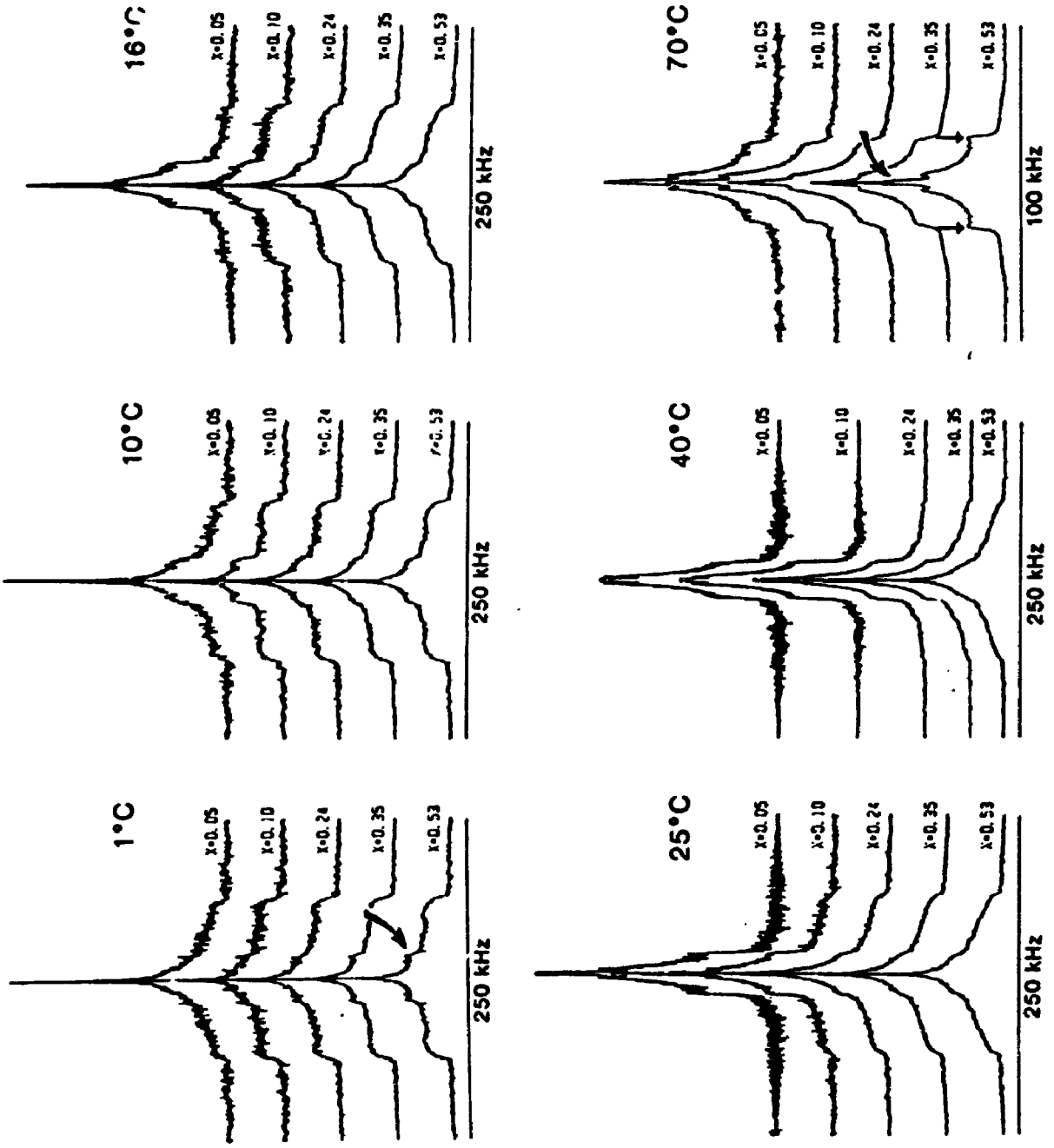

16°C
10°C
1°C
70°C
40°C
25°C
250 kHz
100 kHz

Figure 3.2

Concentration dependence of ^2H NMR spectra for N-stearoyl[d_{35}] galactosyl ceramide (18:0[d_{35}] GalCer) in SOPC at the temperatures 1°C, 16°C and 70°C. Spectra are arranged in vertical groups with glycolipid mole fraction and temperature indicated. Curved arrows indicate features associated with the fatty acid terminal deuteromethyl group for the 1°C (gel phase) and 70°C (fluid phase) 0.05 mole fraction samples.

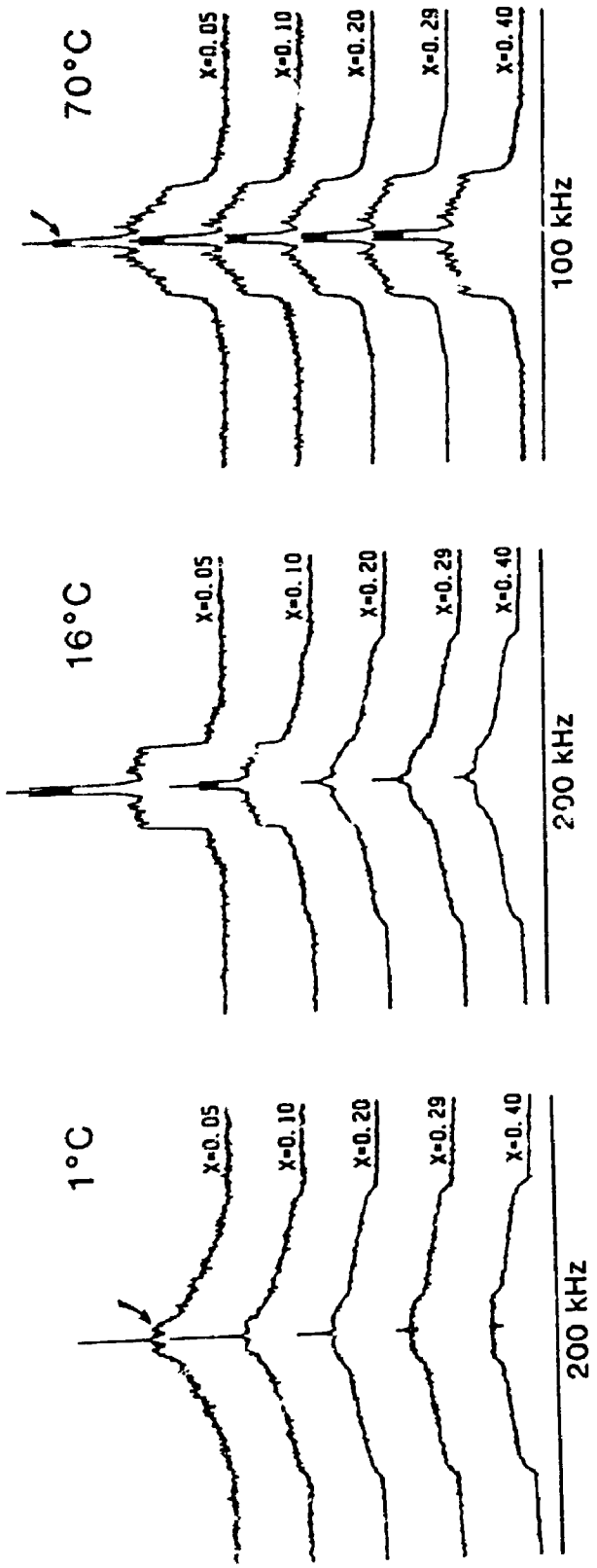
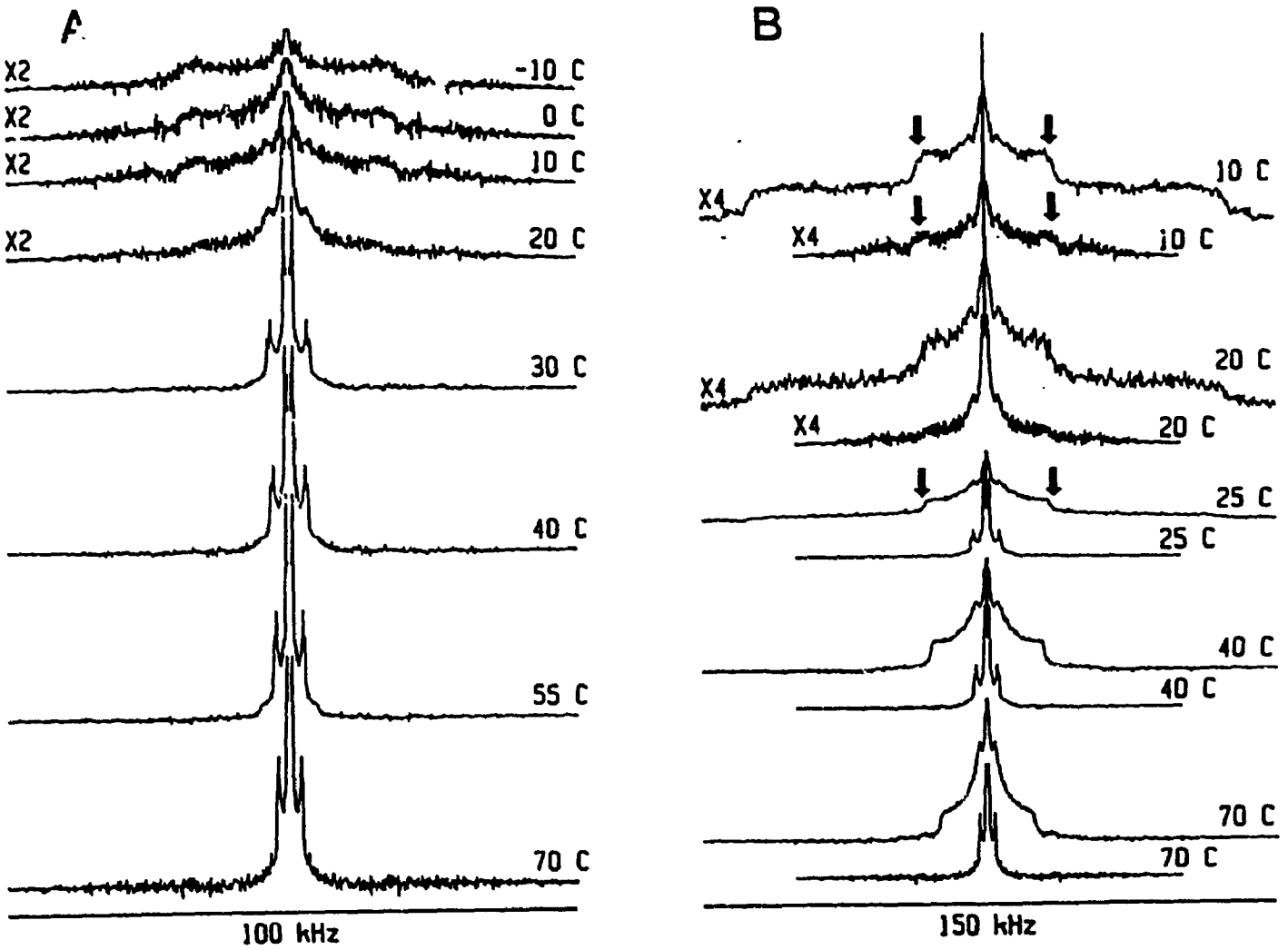

70°C
X=0.05
X=0.10
X=0.20
X=0.29
X=0.40
100 kHz
16°C
X=0.05
X=0.10
X=0.20
X=0.29
X=0.40
200 kHz
1°C
X=0.05
X=0.10
X=0.20
X=0.29
X=0.40
200 kHz

Figure 3.3

Comparison of samples containing N-lignoceroyl-d_7 GalCer (24:0[d_7] GalCer) (ie. specifically labelled on the fatty acid terminal methyl group and the two adjacent methylene groups), with corresponding samples in which the long chain fatty acid was perdeuterated (ie. 24:0[d_{47}] GalCer), at low mol% in SOPC. This comparison makes possible determination of the contribution to the perdeuterated sample spectra, from deuterons toward the methyl terminus of the acyl chain. A) spectra for 24:0[d_7] GalCer in SOPC matrices having a wide range of fluidity properties between 10°C and +70°C. B) direct comparison of selected spectra for the perdeuterated and specifically labelled 24:0 GalCer in SOPC. The spectra of the methyl and last two methylenes appear to account for the narrowest components of the perdeuterated chain spectra. The prominent feature with edges near ± 17 kHz (splitting 34 kHz) in the 24:0 GalCer / SOPC spectra at 10°C is indicated by arrows in B. This value is similar to the plateau splitting in the liquid crystalline phase of 24:0[d_{47}] GalCer in SOPC (Figure B at 25°C) in the same mixture. Spectra shown for 24:0[d_7] GalCer contained 8 mg of deuterated glycolipid. GSL concentration is 10 mol%.

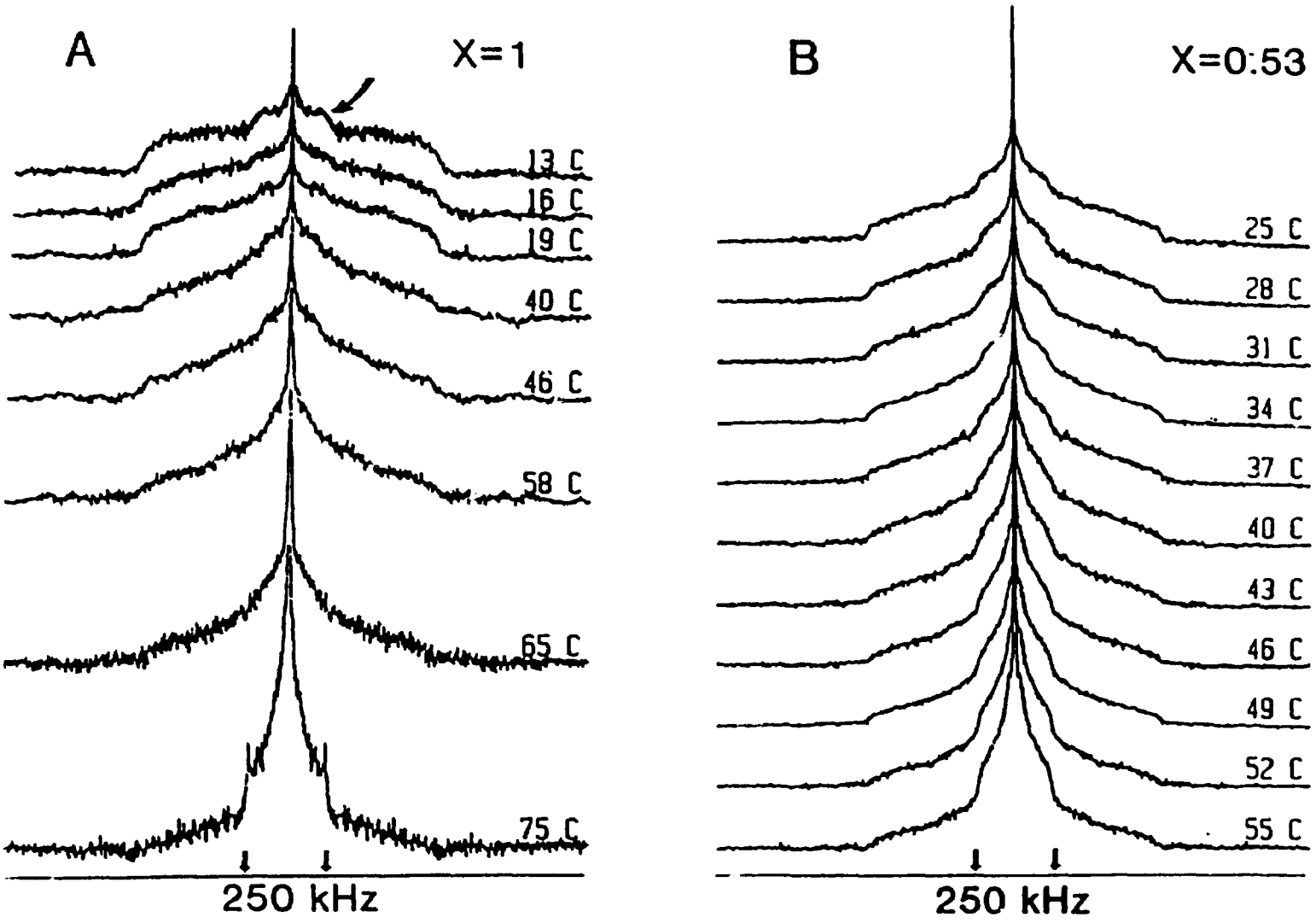

A
X2
-10 C
X2
0 C
X2
10 C
X2
20 C
30 C
40 C
55 C
70 C
100 kHz
B
X4
10 C
X4
10 C
X4
20 C
X4
20 C
25 C
25 C
40 C
40 C
70 C
70 C
150 kHz

GalCer in membranes of homogeneous chain length (C-18 GalCer/SOPC (fig. 3.2) - see below). Also the methyl splitting was small. These characteristics have now been reported for an analogous heterogeneous system containing (18 carbon) fatty acid deuterated DSPC at low concentration in a (14 carbon) DMPC host matrix (Morrow et al., 1991). Further insight into the origin of this intense signal near the centre of the spectrum came from the spectrum of the selectively deuterated GSL (C-24 d_7 GalCer) system. As can be seen, this feature arose from the terminal deuterons of the long chain fatty acid (fig.3.3). The spectral splittings are observed to increase progressively up the chain. (fig. 3.3). This suggested that the extra length of the GSL fatty acid, over that of the shorter chain host matrix, was associated with low orientational order. Upon further examination of the spectra (fig. 3.1), particularly when comparing those at 1°C for X=0.05 and x=0.53, two distinct ordered phase spectra were noted. One was associated with low concentrations of GalCer in SOPC (G1) while the other seemed to be nearly pure GalCer (G2). In the spectra of the C-24 GalCer/SOPC mixtures characteristic G2 spectral features are blended in (in different proportions) with the features of the liquid crystalline spectra or the G1 ordered spectra (Fig. 3.1). The G1 spectrum was characterized by a build-up of intensity near the centre of the spectrum whereas G2 displayed a prominent feature with a width of approximately 34kHz (fig. 3.1). The

selectively deuterated GalCer confirmed the identity of these features (fig. 3.3). As can be seen in fig. 3.3, the large build up of spectral intensity near the centre of the G1 ordered spectra, and the 34kHz feature of the G2 phase, stem from the fatty acid terminal deuterons. This large buildup of intensity in the G1 phase indicates that the extra length of the long chain fatty acid was accommodated by some disordering near the methyl terminus. In contrast, the larger splitting of the G2 spectra most likely results from the terminal portion of the fatty acid chain partially interdigitating with the opposing monolayer. This behaviour has been described for mixed chain phosphatidylcholine, sphingolipid systems and GSL systems (Davis and Keough, 1985; Bloom et al., 1981,Bunow and Levin, 1980; Levin et al., 1985; Huang and Mason, 1986; Boggs and Mason, 1986; Mattai et al., 1987; Florio et al., 1990). The identity of the G2 spectra was confirmed with experiments using pure perdeuterated C-24 GalCer (fig. 3.4). A calculation of the relative area that the shoulders occupy (approx. 34kHz) confirmed that they are consistent with the assignment of the methyl deuterons. This was in agreement with studies using the selectively deuterated (C-24 d_3) GalCer system. The very small build up of intensity near the centre of the pure perdeuterated C-24 GalCer ordered phase spectra likely represented non-equilibrium behaviour. A very narrow central peak (<5kHz), whose size varied with sample history, accompanied the more ordered spectra as the

Figure 3.4.

Temperature dependence of ^2H NMR spectra for: A) pure N-lignoceroyl[d_{47}] GalCer in hydrated bilayer form, and B) N-lignoceroyl[d_{47}] GalCer in SOPC at glycolipid mol fraction x=0.53. The pure GSL sample comprised 7 mg of lipid dispersed in an excess of buffer, frozen and then warmed to 90°C twice before being incubated at 90°C for 1 h. The sample temperature was then lowered to 75°C in several steps over a 6 h interval, following which it was transferred to the NMR probe which had been preheated to 75°C. Each spectrum in A is an average of between 12000 and 36000 transients collected with a repetition time of 0.6 s. Cooling of this sample from 75°C to 50°C proceeded at an effective rate of about 0.6°/h. Vertical arrows indicate ±17 kHz on the frequency axis. The curved arrow in 2A indicates the methyl feature referred to in the text.


A
X=1
13 C
16 C
19 C
40 C
46 C
58 C
65 C
75 C
250 kHz
B
X=0:53
25 C
28 C
31 C
34 C
37 C
40 C
43 C
46 C
49 C
52 C
55 C
250 kHz

temperature decreased. This also likely represented non-equilibrium behaviour. Another feature noted was the liquid crystalline spectrum seen at 75°C (fig. 3.4). N-lignoceroyl GalCer has a main transition temperature of approximately 82°C therefore liquid crystalline features would not be expected at 75°C. This observation in the pure perdeuterated system likely reflects supercooling (Reed and Shipley, 1987) and the well known effect of perdeuteration, namely the lowering of the main phase transition temperature.

With the spectral features which characterize the 3 phases identified, a phase diagram was constructed. The spectra observed from any system would consist of superpositions of these characteristic spectra. The blending of spectra to produce the observed spectrum is quantitatively dependent on the relative amounts of each phase present. The technique of spectral subtraction allows the separation of spectral components arising from each phase (see below).

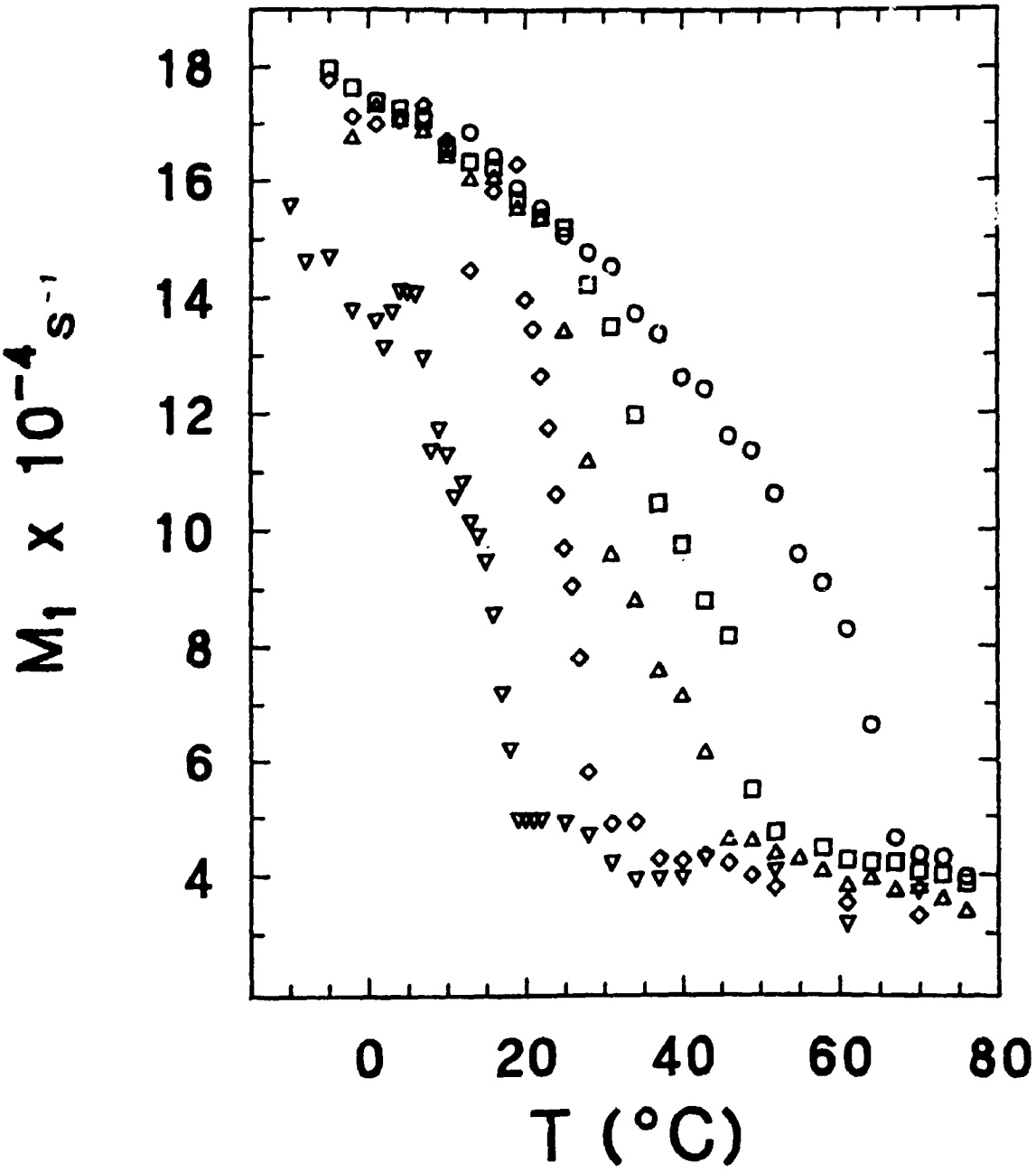
In order to acquire a preliminary idea of what the phase boundaries may be, first moment analysis of the spectra representing samples at different compositions was performed. The first moment, M1, is a weighted average of the splittings (Davis, 1983). M1 is directly proportional to the orientational order parameter for the fatty acid chains:

$$S_{CD} = \overline{<\tfrac{1}{2}(3\cos^2\theta_{CD} - 1)>}$$

where θ_{CD} is the angle between the CD bond and the bilayer normal (Davis et al., 1983). Averages include contributions from the whole length of the deuterated fatty acid. Plots of M1 vs temperature for different concentrations of perdeuterated C-24 GalCer in SOPC are shown in fig. 3.5. The calculations and the plot itself were done by Dr. M. Morrow. The point at which M1 started to greatly increase from the narrow range at high temperatures was indicative of the liquidus temperature. The liquidus curve ran from approximately 20°C at x=0.05 to 65°C at x=0.53. It will meet the SOPC axis at the main transition temperature of 6°C and the GalCer axis at approximately 80°C. The convergence of the values, for each sample at lower temperatures, suggested an approximately horizontal boundary within this temperature range. The high M1 values at low temperature indicated ordered phases. Specifically, the very high M1 values observed for the samples with the highest GSL concentrations suggested a very highly ordered system (G2 or G1+G2). The lower M1 value, at low temperature, displayed by the sample with the lowest GSL concentration overall suggested a less ordered phase (G1). This was consistent with the qualitative observations of the spectra (as previously discussed - see fig. 3.1). Examination of the spectra in fig. 3.1 for the GalCer/SOPC systems revealed

Figure 3.5

Temperature dependence of the first spectral moment, M_1, for N-lignoceroyl[d_{47}] GalCer in SOPC at mole fractions x=0.53 (○), x=0.35 (□), x=0.24 (△), x=0.10 (◇), and x=0.05 (▽).


18
16
14
12
10
8
6
4
$M_1 \times 10^{-4}$ s^{-1}
0
20
40
60
80
T (°C)

the G1 feature which seems to be predominant at 4°C for x=0.05, while that for x=0.24, x=0.35 and x=0.53 seem to have both G1 and G2 features. Between 4°C and 20°C, temperatures in which the SOPC matrix would be above its main transition temperature, G1 and G2 phases are likely indicated at the higher concentrations tested. At the two lower concentrations, a superposition of G1 and liquid crystalline features was apparent.

Once the spectral features that characterize each phase were defined and a crude indication of the phase boundaries was obtained, the technique of spectral subtraction was used to refine the boundary lines further. One small complication present was the fact that the 34kHz methyl feature of the G2 phase overlapped the splitting of the shoulder region of the liquid crystalline phase. Clarification of this was achieved with the observation of the feature's intensity as it varied with temperature - the liquid crystalline feature should increase with temperature while the G2 phase should do the opposite. This was demonstrated in fig. 3.4 for X=0.53 between 55°C and 25°C. The selectively deuterated d_7-GalCer system confirmed the feature's identity (fig. 3.3). The technique of spectral subtraction has been used to define phase boundaries in the past (Huschilt et al., 1985; Vist and Davis, 1985; Morrow et al., 1991). The principle that this technique is based on suggests that, in a region in which two phases

coexist, the corresponding spectrum of that sample would be a superimposition of the two endpoint spectra. The endpoint spectra stem from the intersection of the isothermal tie line with the boundary of the corresponding two phase region of the phase diagram. The relative contributions of the two endpoint spectra are determined by the lever rule which relates the position of the sample temperature and composition on the phase diagram with the relative positions of the endpoint spectra on the phase diagram boundaries. After the spectra are normalized and the attributes of the endpoint spectra are identified, it was then possible to determine what fraction of one spectrum must be subtracted from another to give an endpoint spectrum. The two spectra that are being manipulated are at the same temperature. After the determination of the fraction of one spectrum needed to be subtracted from the other to produce an endpoint spectrum, it is then possible to calculate the endpoint composition. These manipulations were done by Dr. M. Morrow and D. Lu. The resulting phase diagram is shown in fig. 3.6. The vertical error bars depict the onset of the two phase coexistence during sample cooling as judged by the features of the resulting spectra and the behaviour of M1 vs temperature. The horizontal error bars represent the range of subtractions that would produce acceptable endpoint spectra for the samples employed. For further detail on this please see Morrow et al. (1992).

Figure 3.6

Proposed phase diagrams for A) N-stearoyl galactosyl ceramide (18:0 GalCer) and B) N-lignoceroyl galactosyl ceramide (24:0 GalCer) in SOPC. The structures of the glycolipids involved are illustrated as inserts below their corresponding phase diagrams. Vertical error bars represent estimated range of uncertainty in boundary crossings determined by inspection of spectra and consideration of M_1. Horizontal bars indicate the range of experimental values obtained using spectral subtraction techniques with various spectral combinations. The structures of the glycolipids are indicated as inserts to their corresponding plots.

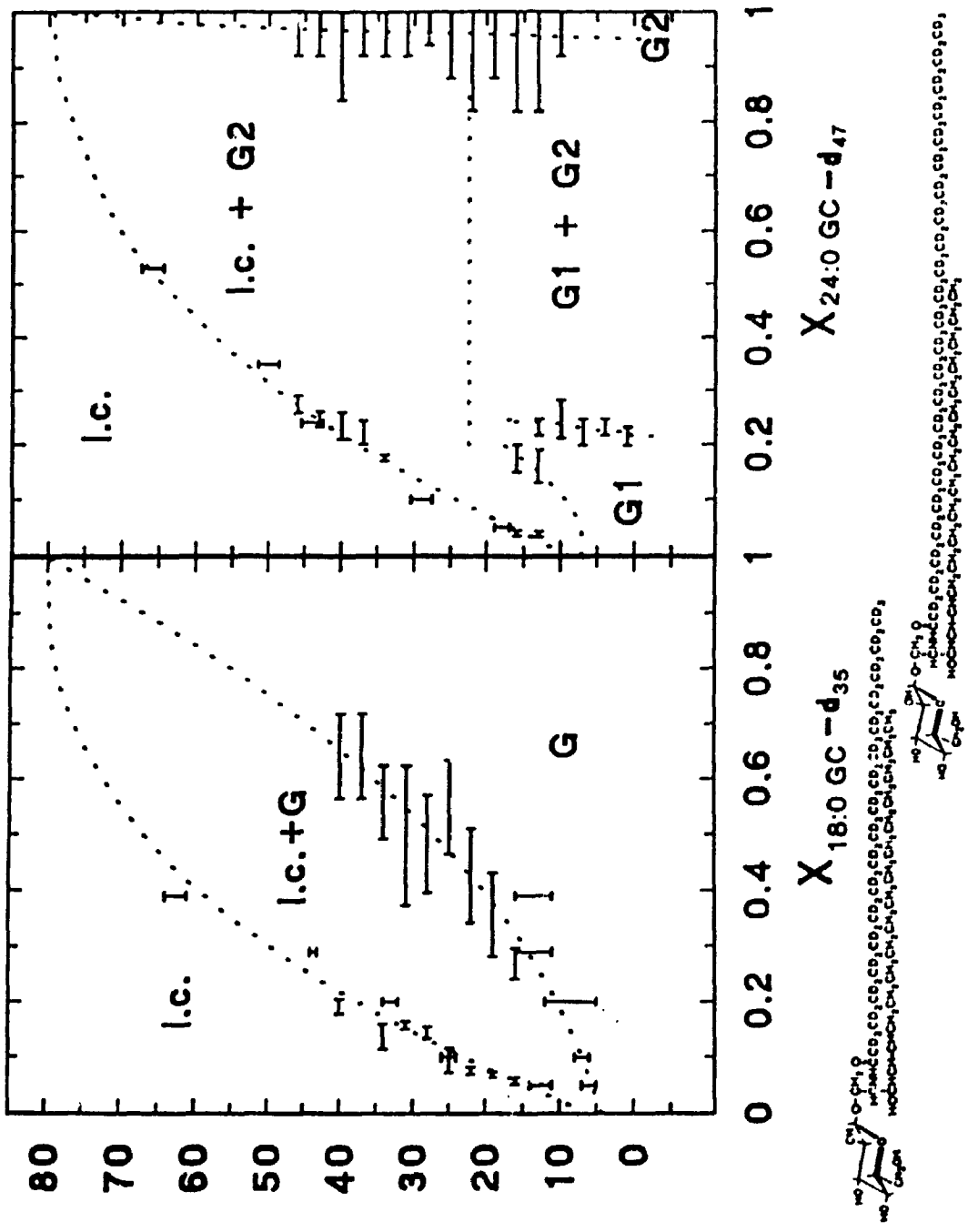
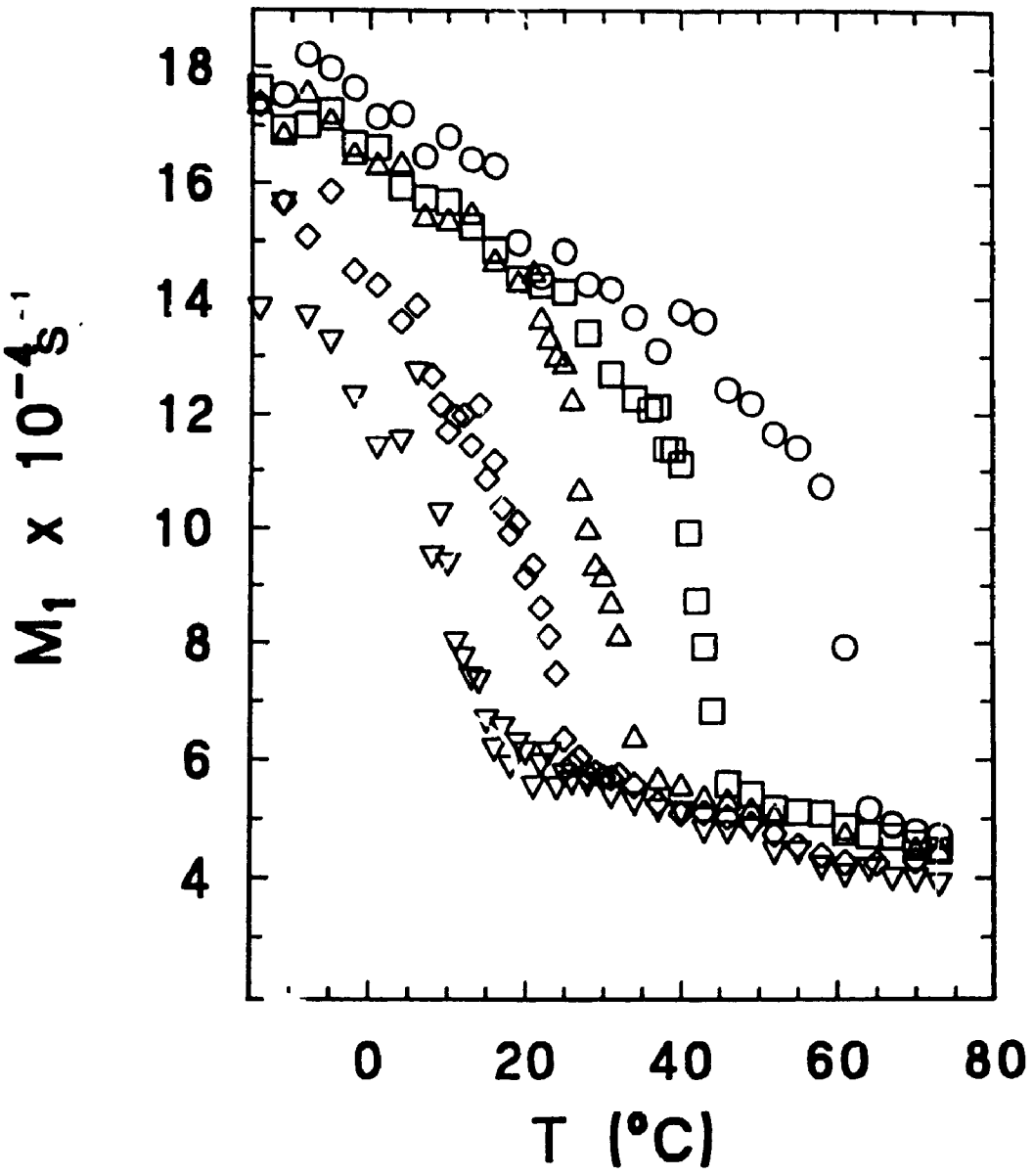

T (°C)
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
l.c.
l.c.+G
G
l.c.
l.c. + G2
G1 + G2
G1
G2
$X_{18:0\ GC-d_{35}}$
$X_{24:0\ GC-d_{47}}$

Figure 3.7

Temperature dependence of the first spectral moment, M_1, for N-stearoyl[d_{35}] galactosyl ceramide in SOPC at mole fractions $x = 0.53$ (○), $x = 0.35$ (□), $x = 0.24$ (△), $x = 0.10$ (◇), and $x = 0.05$ (▽).


18
16
14
12
10
8
6
4
$M_1 \times 10^{-4} s^{-1}$
0
20
40
60
80
T (°C)

3.3.2 *N-stearoyl GalCer spectra*

A phase diagram was constructed for the C-18 GalCer/SOPC system using similar logic to that described for the construction of a C-24 GalCer/SOPC phase diagram described above. Several different C-18 GalCer/SOPC samples and sample conditions were used in order to observe systems in which only one phase, and therefore one spectral feature, dominated. This was in an effort to characterize the spectral features that would distinguish one phase from another.

Fig. 3.2 shows spectra of C-18 perdeuterated GalCer in SOPC bilayers at various temperatures and compositions. The spectra at 1°C all displayed the broad lineshapes characteristic of lipids in the gel phase (Seelig, 1977; Davis, 1983; Smith, 1984). At 70°C , all samples displayed liquid crystalline spectra. The spectra, at 16°C, possessed features of both the liquid crystalline and gel phase spectra. The gel phase feature was exaggerated at x=0.40 while the liquid crystalline feature was most prominent at x=0.05. Close examination of the gel phase spectra revealed no evidence than there was more than one gel phase present. It can be seen, however, that GSL chain order increased with GSL concentration. The terminal deuteromethyl feature (fig. 3.2 curved arrows) demonstrated this effectively as its splitting increased with GSL concentration. The graph of M1 for 5 GSL concentrations also showed increasing values with GSL

concentration at a particular temperature (fig. 3.7). This indicated that orientational ordering in gel and liquid crystal phases was greater for samples with higher GSL concentration. This effect had been observed previously by others (Sharom et al., 1976; Tinker et al., 1976; Sharom and Grant; 1977; Bertoli et al., 1981). As in the study with C-24 GalCer, the variation of M1 with sample concentration and temperature served as a guideline for the phase diagram. The temperature range for which M1 rose sharply gave an approximation of the extent of two phase coexistence. The point at which the M1 values start to rise quickly from the gentle slope gave a crude indication of the liquidus line. Spectral subtraction was used, as described above, for the refinement of the phase diagram.

The comparison of the two phase diagrams presented in fig.3.6, indicated that the glycolipid with the fatty acid chain which matches the length of the host matrix (C-18) was more miscible than the long chain analogue in the gel phase. Above x=0.2, the gel phase for the C-18 species was miscible in SOPC while gel phase immiscibility was seen for the long chain species. Below x=0.2, the behaviours of the two GalCer species were very similar. The liquidus curve was virtually identical for both systems. The phase diagram determined by us for 24:0 GalCer in SOPC is reminiscent of that found by Shipley and coworkers for GalCer with 16:0 fatty acid in DPPC: the latter showed a horizontal solidus above GSL mole fraction 0.2,

solid phase miscibility below this GSL concentration, and peritectic behaviour at GSL mole fractions in the range 0.2-0.3 (Ruocco et al., 1983). A phase diagram for GalCer with longer chain (24:0) fatty acid in the same (DPPC) matrix did not show solid phase miscibility at low GSL concentrations (Gardam & Silvius, 1989). This apparent difference in longer chain GalCer is in the same direction seen in the present work.

3.4 **CONCLUSIONS**

The characteristics and phase behaviour of two similar systems were compared. The first system was comprised of a GalCer species with a fatty acid chain that was longer than that of the host matrix. In the second system, the length of the fatty acid chain was the same as that of the host matrix. A range of temperatures and concentrations were studied. It was found that the fluidus lines for the two phase diagrams of the GalCer systems were superimposable within experimental error. Also, the phase behaviours of the two species were very similar at low concentrations. This is similar to the observation that, at low concentrations, C-24 GalCer did not show any more tendency to segregate laterally in DPPC bilayers than did an analogue with a shorter fatty acid (Gardam and Silvius, 1989). This suggests that fatty acid chain length did not seem to be a significant factor in GSL phase separation in highly fluid phosphclipid membranes at low

concentration. Differences between the C-18 and C-24 species could be seen in the ordered phases. The C-24 species showed two types of ordered phase spectra, which reflected ordered phase immiscibility at intermediate GSL concentrations. One of these was designated G1, an ordered phase rich in SOPC. The other ordered phase was designated G2, which appeared to be nearly pure C-24 GalCer. Only one ordered phase spectrum was seen for the C-18/SOPC system over the concentration range studied. Thus it appears that the fatty acid chain length of GSL is a driving force in lateral segregation in the ordered phases.

In general, it was evident that increasing GSL concentration in SOPC membranes resulted in increased orientational order, regardless of whether the system was in the gel or liquid crystalline phase. This observation was a reflection of that seen in other glycolipid/phospholipid systems (Sharom et al., 1976; Tinker et al., 1976; Sharom and Grant, 1977; Bertoli et al., 1981; Uchida et al., 1981). This likely reflected the fact that GSL phase transition temperatures tend to be 40-80°C higher than those of phospholipids (Curatolo and Jungawala, 1985; Lee, 1977a; Davis and Keough, 1985).

CHAPTER 4

THE INFLUENCE OF THE CERAMIDE FATTY ACID STRUCTURE ON THE BEHAVIOUR OF THE CARBOHYDRATE HEADGROUP OF GALCER

4.1 INTRODUCTION

As previously mentioned, there has been a great deal of speculation about the role of the single fatty acid, on the ceramide moiety of GSLs, in presentation of the sugar headgroup at the membrane surface. It has been widely supposed that the orientation and behaviour of the sugar headgroup are factors in the control of the GSL behaviour in membranes (Curatolo, 1987a). In nature, the fatty acid on the ceramide portion of the GSL tends to be heterogeneous. GSLs frequently possess α-hydroxylated fatty acids and fatty acids that are up to 24 carbons in length (Hakomori, 1981; Thompson and Tillack, 1985; Curatolo, 1987c). Phospholipids are somewhat different in that their fatty acids tend to be between 16 and 18 carbons in length and are not hydroxylated. *cis*-9,10- Unsaturation is seen in the fatty acids of phospholipids and GSLs. Glycolipid fatty acid chain length and α-hydroxylation have been shown to influence GSL antigenic properties (Hakomori, 1986).

Some work has been done on the effects of fatty acids on the behaviour of GSLs. It has been shown that the nature of the fatty acid

influences the gel-to-liquid crystalline phase transition of pure GSL species, although less so than is seen with glycerolipids. The main transition temperatures for N-α-hydroxystearoyl, N-stearoyl and N-lignoceroyl-GalCer are approximately 70°C, 83°C and 83°C respectively (Curatolo and Jungalwala, 1985). N-oleoyl-GalCer exhibits a main transition to a metastable phase at 44.8°C which converts over roughly 24hr to a new gel phase with a main transition of 55.5°C over time (Reed and Shipley, 1989). Thus, characteristics of the fatty acid chain have been speculated to have the potential to control the lateral arrangement of GSLs in a membrane environment (Hakomori, 1986; Kannagi et al., 1983; Lampio et al., 1986; Curatolo and Neuringer, 1986).

There has been much conjecture as to what the GSLs, with varying fatty acid characteristics, are actually doing in the membrane. It has been speculated that GSLs with long-chain fatty acids protrude further from the membrane surface than do shorter chain analogues. Evidence supporting this idea came from antibody studies which demonstrated that a long-chain fatty acid on the ceramide portion of GalCer could distinctly increase antibody receptor function of GalCer as compared with a shorter chain analogue, within a membrane environment (Alving et al., 1980; Schichijo and Alving, 1985). This was a situation in which the GalCer fatty acid was much longer than that of the host matrix. Phosphatidylcholine systems, with

the phospholipid either having short or long chain fatty acids, containing GalCer were also compared. The relative fatty acid chain length between the GSL and host matrix was demonstrated to be a critical factor in determining antibody reactivity in these experiments. The same observation has also been reported in studies involving the sulphated derivative of GalCer (Crook et al., 1986). Thus, there may be greater access of the carbohydrate residue to the antibody as compared to the shorter chain analogue. Macromolecule binding characteristics to lipids have also been shown to be affected by cholesterol content and temperature (Brulet and McConnell, 1977; Balakrishnan et al, 1982; Utsumi et al, 1984; Stanton et al, 1984; Mehlhorn et al 1988; Stewart and Boggs, 1990). It has been pointed out in these studies that these factors have the potential to alter lipid recognition site dynamics, as well as orientation and lateral distribution.

Glycolipid crypticity is also influenced by fatty acid hydroxylation (Kannagi et al., 1983, Hakomori, 1986). It has been suggested, from X-ray studies, that the D-α-hydroxyl group of GalCer can hydrogen bond to its galactose headgroup (Pascher and Sundell, 1977). However, using Raman spectroscopy, it was demonstrated that in hydrated bilayers of GalCer, the intramolecular hydrogen bonding is different from that observed in X-ray studies (Bunow and Levin, 1980). Thus, whether the factors, involved in hydrogen bonding in the studies of pure species, are also active in the

hydrated heterogenous membrane context was unknown.

Using ^{2}H-NMR, the effect of fatty acid chain structure on the behaviour of the carbohydrate headgroup of GalCer, in a phosphatidylcholine matrix, was examined in the present study. We specifically addressed the question of communication between the sugar headgroup and the acyl chain regions of GalCer in a membrane environment. N-(stearoyl)-GalCer (C-18), N-(lignoceroyl)-GalCer (C-24); N-(oleoyl)-GalCer(C-18:1); N-(D-α-hydroxy)stearoyl-GalCer(D-α-OH) and N-(L-α-hydroxy)stearoyl-GalCer (L-α-OH) were studied at 10 mol% in a POPC matrix. POPC is representative of a typical phosphatidylcholine found in membranes and has a phase transition of -3°C (Davis and Keough, 1985). The nonperturbing deuterium probe was covalently attached to the galactose headgroup of GalCer, replacing a proton on the residue, thus allowing direct monitoring of the behaviour of the sugar headgroup.

4.2 MATERIALS AND METHODS

4.2.1 *Source of Materials*

1-palmitoyl-2-oleoyl-3-sn-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, Birmingham, AL; and was used without further purification. Galactosyl ceramide (GalCer) from a beef brain source was

also from Avanti Polar Lipids. Lyso-GalCer (ie. GalCer with the fatty acid removed) was produced from the above material by hydrolysis in refluxing butanolic KOH (Taketomi and Yamakawa, 1963) and was ninhydrin positive. The lyso-GSL was applied to a column of Bio Rad silicic acid 200-400 mesh, and eluted with a gradient of methanol in chloroform. Isolated materials co-chromatographed with known material on Merck silica gel 60 thin layer chromatography plates eluted with 65:15 $CHCl_3/CH_3OH$ and developed with sulphuric acid/ethanol.

4.2.2 *Synthesis of galactosylceramide species*

Galactosyl ceramides with α-hydroxy fatty acids were synthesized via their α-acetoxy analogues as follows. α-acetoxy stearic acids were prepared from the corresponding α-bromo intermediates, which had been generated by reacting stearic acid (Aldrich) with liquid bromine in the presence of PCl_3, followed by hydrolysis of the acid chloride product (Allen and Kalm, 1967). Conversion to the corresponding α-acetoxy esters was accomplished by refluxing with anhydrous sodium acetate in glacial acetic acid (Guest, 1947). The mixture of stearic acid α-acetoxy esters was purified on a silicic acid column eluted with $CHCl_3$, and converted to the acid chloride form by refluxing with excess $SOCl_2$ (Kopaczyk and Radin, 1965). These reactions were followed by TLC on silicic acid plates eluted with hexane/diethyl

ether/formic acid (70/30/1). The α-acetoxy ester was identified by ^1H NMR in C^2HCl_3. The fatty acid chlorides were coupled with lyso-GalCer, followed by hydrolysis of the acetate group (Pascher, 1974). D- and L-α-hydroxy stereoisomers of the GSL were separated using a silicic acid column eluted with a $CHCl_3/CH_3OH$ gradient, the L- isomer being significantly faster-running in the solvent system described (Pascher, 1974). Semi-synthetic non-hydroxy and D-α-hydroxy GalCer behaved similarly, but not identically on TLC, to natural beef brain GalCer non-hydroxy and D-α-hydroxy fractions respectively - the semi-synthetic species running slightly slower than their corresponding natural mixtures, which included longer fatty acids, as reported previously (Karlsson and Pascher, 1971).

N-stearoyl GalCer and N-lignoceroyl GalCer were generated in a similar fashion to the above hydroxylated species: hooking up the appropriate fatty acid (as its acid chloride) to lyso-GalCer. N-oleoyl GalCer was generated by coupling lyso-GalCer with oleic acid in the presence of dicyclohexylcarbodiimide and pyridine (Sharom and Grant, 1975). This method was used instead of the procedure involving the acyl chloride to avoid destruction of the *cis* 9,10 unsaturation in oleic acid.

4.2.3 *Deuteration of galactosylceramides*

Deuteration of the GalCer carbohydrate residue at C_6 was achieved

by oxidation of the C_6 hydroxymethyl group on the various GalCer species with galactose oxidase/horse radish peroxidase, followed by reduction of the resulting aldehydes with $NaBD_4$ (Radin and Evangelatos, 1981). Purification was as previously described using chloroform/methanol/water partitions (Radin, 1972).

4.2.4 *Preparation of samples for ^{2}H-NMR*

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio (10 mol% glycolipid) in 3:1 $CHCl_3/CH_3OH$, and removing the solvent using a rotary evaporator and a water bath at 50° C. Resultant films were further dried under vacuum (rotary pump) for 3 h at 22° C. Liposomes were generated by initial hydration of such films with deuterium depleted water (MSD Isotopes Ltd) buffered with 10 mM phosphate at pH 7.4. Samples were subsequently lyophilized three times from a volume of 300 µl of deuterium depleted water, and subjected to eight freeze-thaw cycles following the final hydration step. Total lipid used per sample was typically 100-200 mg (of which 10-14 mg (11.6-16.2 µmol) was deuterated), and the total final volume was 500-700 µl. All samples were incubated at 60° C to ensure diffusional equilibrium within the bilayer.

4.2.5 *Acquisition of spectra*

^{2}H NMR spectra were acquired at 30.7 MHz on a "home-built" spectrometer operated by a Nicolet 1280 computer (Chapter 2; Singh et al., 1992a). Spectra were recorded using the quadrupolar echo pulse sequence (Davis et al, 1976) with full phase cycling of the rf pulses (Perly et al, 1985) and quadrature detection. The $\pi/2$ pulse length varied from 5 to 6μs (10 mm solenoid coil), pulse spacing was 60 μs, and recycle time was 80 ms. Spectra were not folded about the Larmor frequency. The 90° oriented sample ("depaked") spectra were calculated from the powder spectra as described previously (Bloom et al, 1981).

4.2.6 *Freeze Fracture*

Samples for freeze-fracture electron microscopy were withdrawn directly from the samples prepared for ^{2}H NMR and thus had the same history. The pure POPC control specimen was prepared without deuterated glycolipid and subjected to manipulations identical to those for the NMR samples. Prior to the 'quenching' step that preserves sample structure for heavy metal shadowing, samples were incubated under conditions designed to approximate as closely as possible the conditions under which NMR was performed. This involved placing small droplets of the liposome suspensions on gold alloy planchets in 6 mm holes drilled horizontally into a 6x6x22 cm

brass block. It was in turn immersed to just below the level of the sample hole in a water bath held several degrees above the NMR temperature to allow for slight cooling that occurs during sample transfer to the cryogenic bath. The bath itself was maintained in a temperature-controlled room at 35-37°C. The atmosphere surrounding the brass block and samples was saturated with water vapour from the bath. Samples were removed from the drilled brass block with very fine forceps and frozen instantly in a slurry of freon 22 cooled in liquid nitrogen. Frozen samples were freeze fractured at -110°C, and platinum shadowed in a Balzers BAF 301 apparatus equipped with electron beam guns. Replicas were cleaned initially in $NaClO_4$, rinsed with distilled water, and immersed in 1/1 acetone/ethanol for 1 h to remove residual lipid. Replicas were picked up on 400 mesh copper grids and viewed in a Phillips EM300 electron microscope. The freeze fracture investigations of the lipid samples used in this study were performed by Ms. K. Barber of our laboratory.

4.3 **RESULTS AND DISCUSSION**

4.3.1 *Background*

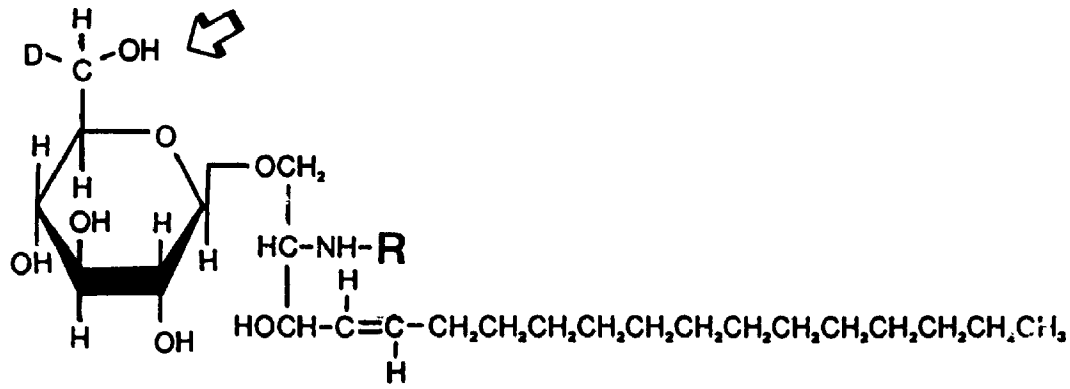
Five different GalCer systems were studied with the only variable between samples being the fatty acid chain structure of the ceramide

backbone (see Fig. 4.1). Each sample contained a single GalCer species at 10 mol% in a POPC host matrix. The fluidus in a published phase diagram of POPC/natural GalCer, at 10 mol% GalCer, occurred at about 30°C (Curatolo, 1986). The monitor of the headgroup motion via the deuterium label attached to the sugar would reflect its behaviour. With this experimental design, it was intended that the role of the fatty acid in the behaviour of the headgroup would be isolated.

The galactose oxidase labelling procedure of the galactose sugar residue of GalCer produces two spectroscopically inequivalent stereoisomers. The *pro-R* and *pro-S* diastereomers each give distinct signals which are a reflection of different average orientations of the carbon-deuterium bonds. Thus, the resulting spectrum from this type of sample is a superposition of two quadrupolar powder spectra. The two monodeuterated stereoisomers are produced in equal quantities as a result of the random replacement of the C_6 protons. The spectrum from each isomer would be of equal intensity (1:1 ratio - a reflection of the isomer populations). Hydrated bilayers of pure N-(stearoyl)-GalCer, deuterated in this fashion, have been previously studied (Skarjune and Oldfield, 1979) with the properties, described above, being observed. Pure hydrated lactosyldiglyceride, deuterated on the exocyclic C_6 position of the galactose

Figure 4.1

Chemical structures for the deuterated glycolipids studied in this work. The mono-deuterated exocyclic hydroxymethyl group of the galactose headgroup is indicated (arrow) - two stereoisomers are possible. "R" refers to the fatty acid attached in amide linkage: from top to bottom, the 24-carbon saturated species (lignoceric acid), and the 18-carbon species (stearic, oleic, and α-OH-stearic acids respectively).



R =

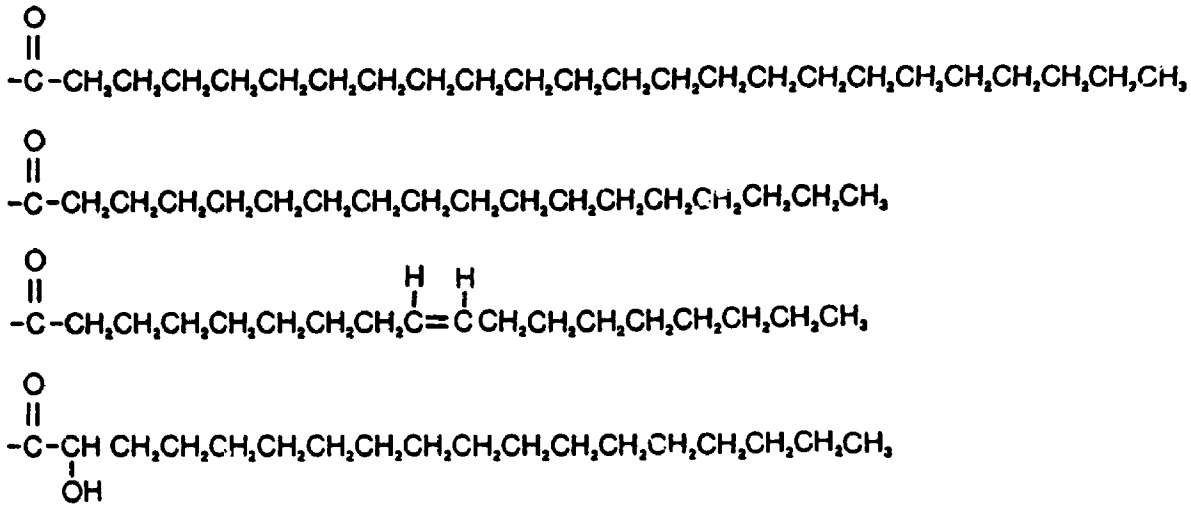


Figure 4.2

^{2}H NMR powder spectra at 65°C for low concentrations of headgroup-deuterated GalCer in phospholipid bilayers, as a function of ceramide fatty acid composition. Deuteration was at C_6 of the galactose residue as indicated in Figure 4.1. In each case the glycolipid was dispersed at 10 mol% in unsonicated multilamellar vesicles of 1-palmitoyl-2-oleoyl phosphatidylcholine. A) N-stearoyl [d_1] GalCer (depaked spectrum on right). B) N-lignoceroyl [d_1] GalCer. C) N-oleoyl [d_1] GalCer. D) N-(D-α-OH)-stearoyl [d_1] GalCer. E) N-(L-α-OH)-stearoyl [d_1] GalCer. Each sample contained 10-14 mg (11.6-16.2μmol) of deuterated GSL (ca. 50% labelling at each of the *pro-R* and *pro-S* C_6 positions).

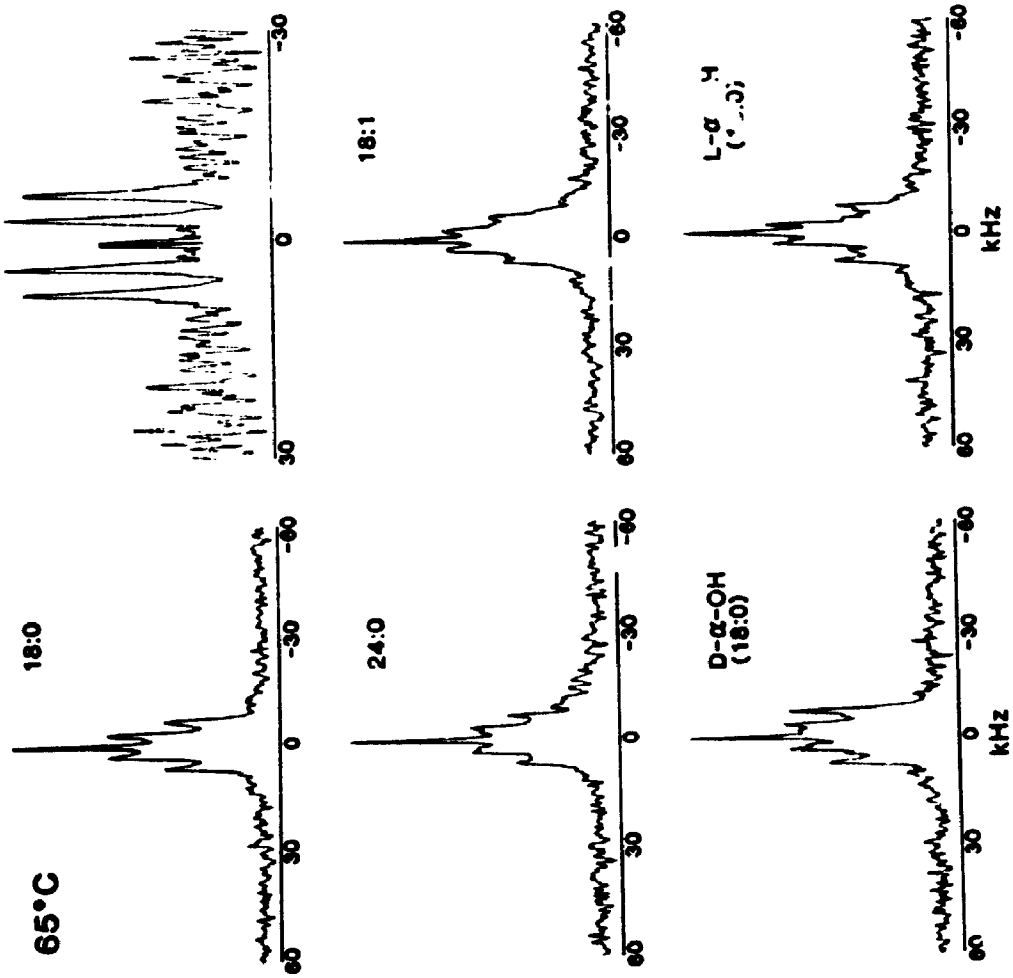
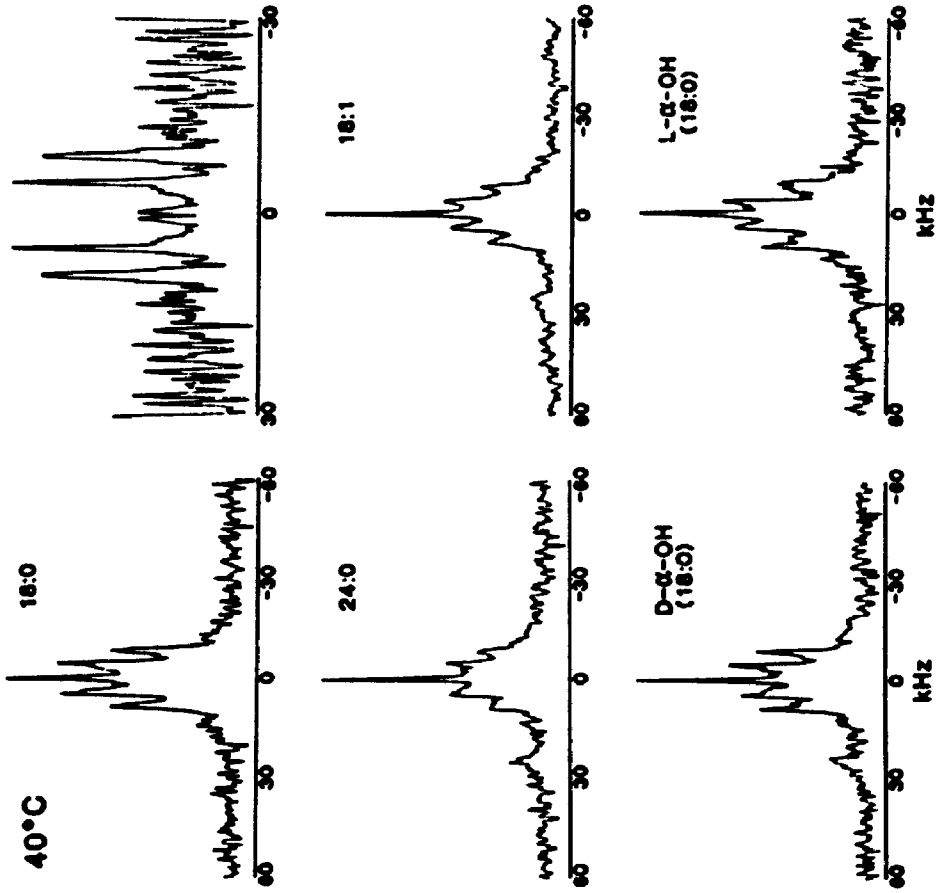

65°C
18:0
24:0
18:1
D-α-OH
(18:0)
L-α-OH
(18:0)
kHz
kHz

Figure 4.3

^{2}H NMR powder spectra at 40° C for low concentrations of headgroup-deuterated GalCer in phospholipid bilayers, as a function of ceramide fatty acid composition. Samples were those run to generate Figure 4.2. The number of spectral accumulations for each spectrum ranged from 308,000 to 374,000, and was the same for a given sample at 65° C (Figure 4.2) and 40° C (Figure 4.3). A) N-stearoyl [d_1] GalCer (depaked spectrum on right). B) N-lignoceroyl [d_1] GalCer. C) N-oleoyl [d_1] GalCer. D) N-(D-α-OH)-stearoyl [d_1] GalCer. E) N-(L-α-OH)-stearoyl [d_1] GalCer.


40°C
16:0
18:1
24:0
D-α-OH (18:0)
L-α-OH (18:0)
kHz
kHz

residue, has been studied producing spectra reminiscent of the pure GalCer system studied by Skarjune and Oldfield (1979).

4.3.2 *In general*

The powder spectra for the 5 different samples described above are shown in Figures 4.2 and 4.3 . All of the spectra showed characteristics of fluid phase consistent with axially symmetric motional averaging. An example of a simulated 90° oriented (depaked) spectrum for the C-18 sample is also shown. Two powder patterns of roughly equal intensity made up each spectrum shown in Figures 4.2 and 4.3. This was more obvious with inspection of the depaked spectra (some of which are not shown). These results were very similar to those seen previously with studies of pure hydrated GalCer bilayers (Skarjune and Oldfield, 1979).

4.3.3 *Comparison between samples and temperatures*

4.3.3.1 *GalCer species share some behavioural characteristics*

The quadrupolar splittings for the spectra at 40°C and 65°C are shown in Table 4.1. At 40°C, the splittings were the same, within experimental error, for all the samples with the exception of the L-α-OH

sample, which does not occur in nature. Within the 65°C samples, both of the α-hydroxylated samples differed from the rest beyond experimental error. The spectral splittings from these two samples were equal within the bounds of experimental error. The above similarities of the spectra, at a given temperature, strongly suggested that the GSLs in each system were all behaving in a very similar fashion.

Glycolipids, studied using a variety of techniques in various settings, have been proposed to exhibit a number of properties and behaviours which are dependent on the composition of the ceramide backbone. As previously mentioned, Carl Alving and co-workers (1980) examined the effect of the fatty acid chain length and matrix fatty acid chain length on the ability of GalCer to act as a receptor for monoclonal antibodies. It was found that the relative fatty acid chain length on the glycolipid relative to that of the phosphatidylcholine host matrix was important. It has been suggested that a relatively longer fatty acid on the GSL results in the greater accessibility of the sugar headgroup to the antibody via its protrusion above the headgroups of the matrix. The C-18 and C-24 samples directly addressed this situation. Calculations using glycerolipids and GSLs have shown that headgroup conformation and orientation are subject to the distance the carbohydrate is from the membrane as well as the lipid cross-sectional area (Nyholm et al., 1989; Winsborrow and Jarrell, unpublished

results). ^{2}H-NMR is sensitive to very small changes in orientation and to motional dynamics (Skarjune and Oldfield, 1979; Davis, 1983; Seelig and MacDonald, 1987; Jarrell et al., 1986). Any difference in these parameters, between the systems studied, would be reflected in the quadrupolar couplings - $\Delta\nu_Q$. Thus, (see Table 4.1) there appeared to be virtually no effect of acyl chain length on headgroup behaviour.

The two samples with the α-hydroxy fatty acids allowed the examination of the potential intramolecular hydrogen bonding role of this function with the sugar headgroup and in intermolecular hydrogen bonding. Speculation concerning these possibilities arose from X-ray crystallographic measurements (Pascher and Sundell, 1977; Nyholm et al., 1990). Within a membrane context however, the comparison with the results arising from single crystals may not apply. It has been demonstrated that intramolecular hydrogen bonding, in hydrated bilayers of GalCer as studied by Raman spectroscopy, differs from that observed in the X-ray studies (Bunow and Levin, 1990). If the involvement of the α-hydroxy group of the fatty acid played a significant role in hydrogen bonding, an alteration in either the headgroup orientation or perhaps in the lipid phase behaviour was expected. This would be reflected in the quadrupolar splittings from the α-hydroxy samples. Within our binary systems, the spectral splittings from the α-hydroxy samples showed very small differences from those of the non-

hydroxy samples thus implying a minimal amount of change in orientation and lipid dynamics. It is intriguing that fatty acid hydroxylation has been observed to influence GSL receptor function in some cells (Kannagi et al., 1983).

4.3.3.2 *The effect of temperature on headgroup orientation*

Between temperatures, it can be seen that the spectral splittings at the lower temperature were greater than those of the higher temperature (Table 4.1). This difference can be suggested to arise from either a change in headgroup orientation or in the magnitude of motion about the bilayer normal. The resolution of these two possibilities came from the comparison of the ratios of the two splittings from each spectrum. This ratio is a reflection of the conformational equilibrium between the rotameric states of the C_5-C_6 bond in the galactose headgroup (Renou et al., 1989; Skarjune and Oldfield, 1979). The ratios of the outer/inner $\Delta\nu_Q$, for the stereoisomeric C_6 deuterium label in each sample (Table 4.1), were all equal, within experimental error, at both temperatures with the exception of the L-α-OH derivative. This strongly argues that there was no major change in either headgroup orientation or rotamer population. It appeared that a decrease in temperature therefore resulted in an increase in the orientational order of the headgroup.

TABLE 4.1

Temperature and acyl chain dependencies of quadrupolar splittings ($\Delta \nu_Q$) for headgroup-deuterated galactosyl ceramide at low concentration in fluid phospholipid bilayers. In each case the deuteron was located on the exocyclic hydroxymethyl function of the glycolipid galactose residue (Figure 4.1). $^2H_1$ GalCer was assembled at 10 mol% into unsonicated bilayers of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), suspended in 10 mM phosphate buffered saline pH 7.4.

Table 4.1

T(°C)	Glycolipid Fatty Acid	Inner Splitting (±0.5 kHz)	Outer Splitting (±0.5 kHz)	Splitting Ratio (O/I±0.1)
65°C	24:0	7.4	13.9	1.9
	18:0	7.0	13.8	2.0
	18:1	6.5	13.5	2.1
	18:0 D-α-OH	7.6	15.2	2.0
	18:0 L-α-OH	6.1	16.3	2.7
40°C	24:0	10.2	18.0	1.8
	18:0	9.7	17.7	1.8
	18:1	9.2	17.3	1.9
	18:0 D-α-OH	9.9	18.0	1.8
	18:0 L-α-OH	9.0	20.7	2.3

4.3.3.3 *Phase separation at lower temperatures*

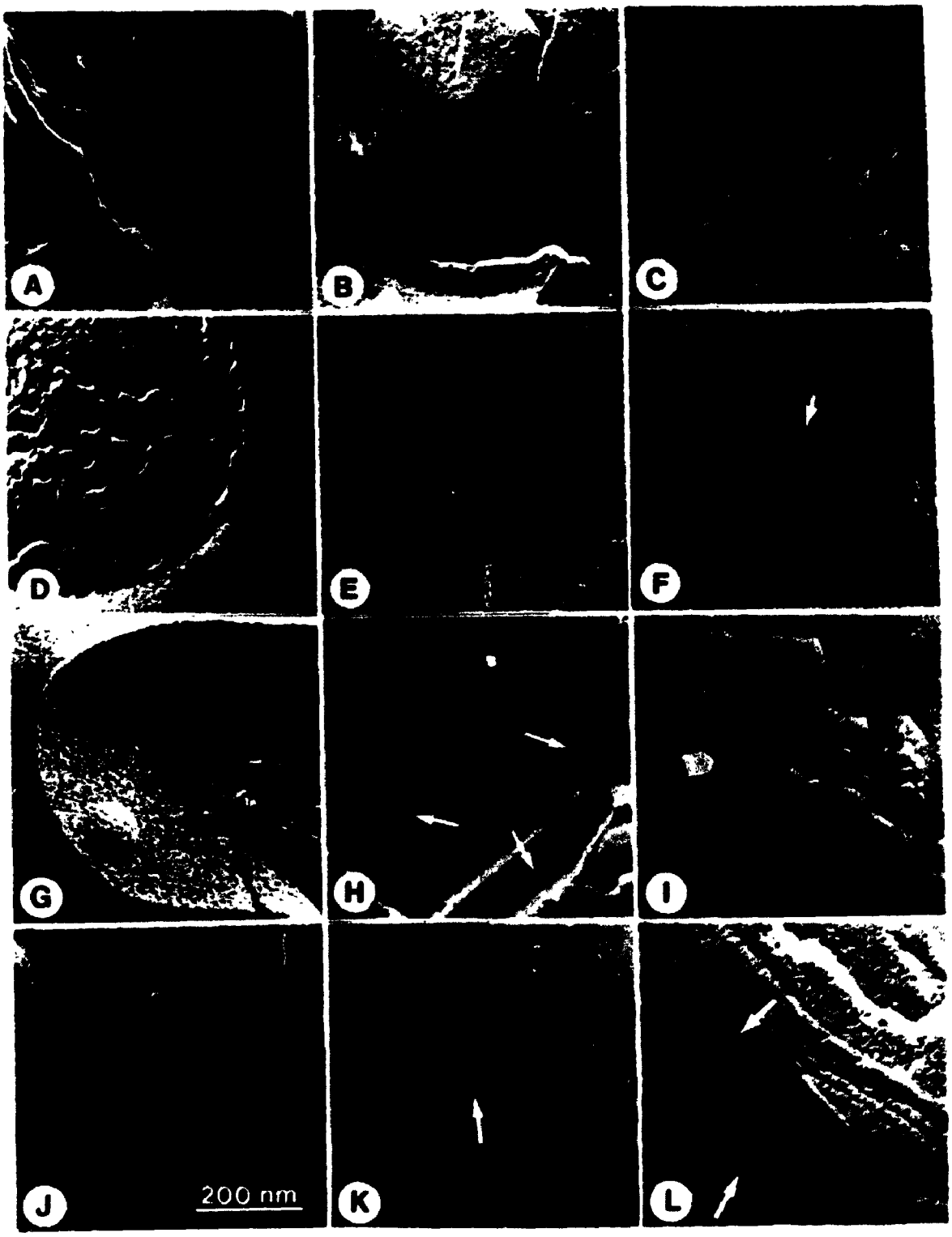
The number of acquisitions for any given sample was the same regardless of temperature. This feature allowed the comparison of the depaked spectral intensities for a given sample between temperatures. This gave information regarding the relative amount of labelled species in the gel phase since any labelled material in a gel phase would not contribute to the liquid crystalline peak intensities. Thus, one may speculate about the amount of GSL in gel phase at one temperature in comparison to another. The aforementioned explanation for a decrease in spectral intensity was most probable but a large change in the transverse relaxation rate was also another consideration in this phenomenon. A reduction in spectral intensity, as the temperature was lowered from 65°C to 40°C is seen for the C-18 and C-24 samples (Fig. 4.2 and 4.3). The spectral intensities of the other samples with the unsaturated and α-hydroxy fatty acids appeared to be similar between temperatures. This was likely an indication of some gel phase separation of the C-18 and C-24 samples at 40°C as compared to 65°C. It should be noted that the signal coming from labelled material in the gel phase would give a unique signal of its own. In this experiment though, the amount of labelled GSL was small, therefore the signal would be weak. The gel phase spectrum would likely be broad and hidden by the liquid crystalline feature as well as the noise in the spectrum. Together, these

considerations would make any gel characteristic virtually impossible to observe.

To further study the physical characteristics of these GSL systems freeze-fracture electron microscopy was employed (Fig. 4.4). This was done by Ms. K. Barber of our laboratory. Freeze-fracture electron microscopy involves the rapid freezing of the sample and, after fracturing, the replication of the exposed face with a heavy metal shadow. Overall, features present in the subject are preserved and may then be viewed using an electron microscope. In pure systems the gel phase, a state in which the lipid is packed in an orderly fashion, can be characterized by a regular rippled appearance while the same system in the fluid phase does not show such a feature (Ververgaert et al., 1973; Grant et al., 1974). Similar features also can be identified in multicomponent systems (Grant et al., 1974; Peters et al., 1984; Mehlhorn et al., 1986; Rock et al., 1990). All of the spectra at 65°C possessed only liquid crystalline features. There was some jumbled-ripple seen which was likely a result of the limitation in the freezing of the sample. Ideally the sample should freeze instantly, but since this is impossible, the lipids are able to diffuse over short distances, during the process of quenching from the temperature of interest, in an effort to assume some orderly gel-phase packing. This effect has been described previously for GSL/phosphatidylcholine systems (Peters et al., 1984). The lipid

Figure 4.4

Freeze-fracture electron micrographs of samples studied by ^2H NMR. Procedures were chosen so that sample conditions immediately preceding freezing were those present during the NMR experiments at 65°C and 40° (see Materials and Methods). 10 mol% N-(D-α-OH)-stearoyl [d_1] GalCer in POPC, corresponding to 65°C (A) and 40°C (B). Pure POPC control without glycolipid (40°C) (C). 10 mol% N-oleoyl [d_1] GalCer in POPC, corresponding to 65°C (D) and 40°C (E). 10 mol% N-stearoyl [d_1] GalCer in POPC, corresponding to 65°C (G) and 40°C (H). The 40° micrograph was selected to show a membrane region with a rigid domain in equilibrium with (more typical) liquid crystal domains: the arrows point from rigid membrane to the domain boundaries. 10 mol% N-lignoceroyl [d_1] GalCer in POPC, corresponding to 65°C (J) and 40°C (I,K,L). K and L were selected to display obviously rigid domains enriched in the 24-carbon fatty acid GSL: arrows point from rigid membrane to the domain boundaries; while the micrograph in I shows a potentially domain-related feature (sharply demarcated circular patches) without obviously rigid nature. F illustrates the appearance of rigid domains (arrow) which can be found in binary mixtures of POPC with the other glycolipids studied at lower temperatures (in this case 5 mol% N-oleoyl GalCer in POPC at 20°C). Magnification x100,000. Bar indicates 200 nm. Shadow direction from bottom to top of page.


A
B
C
D
E
F
G
H
I
J
200 nm
K
L

reorganization that takes place during the fraction of a second that it takes for the sample heat to be transferred to the cryogenic bath (quench), can be reduced by ultrafast freezing (Mehlhorn et al., 1986; 1988; Rock et al., 1990). Inspection of Fig. 4.4 revealed a homogeneous fluid bilayer appearance for the samples containing 10 mol% of either the C-18:1 or the α-hydroxy GalCer species in addition to the pure POPC sample (Fig. 4.4 panels B, E and C respectively). The samples containing either the C-18 or C-24 species at 40°C showed domains characteristic of a highly ordered membrane bilayer in gel phase (Fig. 4.4 panels H, K and L). This feature was also be observed for the C-18:1 and α-hydroxy samples but at lower temperature (Fig. 4.4 panel F). This indicated the existence, based on the published phase diagram for beef brain GalCer (Curatolo, 1986), of gel-phase lipid enriched with GalCer coexisting with fluid domains enriched with POPC. GalCer from beef brain is composed of hydroxylated fatty acids (60%) and a range of saturated fatty and unsaturated fatty acids 16-24 carbons in length (Bunow and Levin, 1980; Gambale et al., 1982). It would appear that a GalCer system with a homogeneous fatty acid on ceramide demonstrates characteristics distinct from that of a naturally occurring mixture of GalCer species in a POPC matrix. The study of POPC/cholesterol mixtures using freeze fracture has also demonstrated coexisting domains (Ververgaert et al., 1973; Grant et al., 1974; Verkleij et al., 1974; Verkleij et

la.,1973; Luna and McConnell, 1977).

One potential limitation in the interpretation of the NMR experiments done in this study stemmed from the fact that the signals observed here, in these studies, represent the majority of the species within the system. The possibility that a small minority of lipid species, in slow exchange with the lipid majority, behaving very differently from the rest of the population cannot be ruled out. Signals that come from a minority behavioural species would likely be hidden by the dominant signal.

4.4 **CONCLUSIONS**

Use of the hydroxymethyl deuterium label on the galactose headgroup of GalCer permitted the direct and sensitive monitoring of headgroup orientation and dynamics. The results from these experiments demonstrated relative headgroup insensitivity to acyl chain characteristics. The α-hydroxy function caused the greatest change among the 5 GalCer species studied, though the differences observed were very small. Thus, it would appear that hydrogen bonding involving the α-hydroxy function was a very minor consideration in headgroup orientation and dynamics. The effect of temperature was confined to changes in lipid dynamics. The saturated C-18 and C-24 species showed a greater tendency to phase separate in POPC than did the α-hydroxylated and unsaturated species.

CHAPTER 5

THE EFFECT OF NATURALLY OCCURRING MEMBRANE ATTRIBUTES ON COMPLEX GSL OLIGOSACCHARIDE BEHAVIOUR

5.1 INTRODUCTION

Features that occur naturally in membranes have been widely held to modulate GSL receptor function and to determine their structural role. Inter- and intramolecular interactions must control GSL conformation and behaviour. In Chapter 4, the simple glycolipid, GalCer, was used as a model to investigate the effect of fatty acid chain characteristics on sugar conformation and behaviour. In this Chapter, the behaviour of the more complex GSLs (gm_1, asialo-gm_1 and globoside) was examined using ^{2}H-NMR, along with the influence of several different factors that occur naturally in membrane environments.

The charged complex ganglioside, gm_1, is a component of neural tissue (Hakomori, 1981; Hakomori, 1986). Its carbohydrate portion is composed of five sugar residues, one of them being the negatively charged N-acetyl neuraminic acid (NANA) (see Fig. 5.1). While the fatty acid of gm_1 is homogeneous, its sphingosine backbone is not: it is composed of 18- and 20 carbon species. Thus effects of the alkyl chain length on the oligosaccharide chain might be observed. We used deuterium probes located within the sugar residues to monitor both gm_1 and

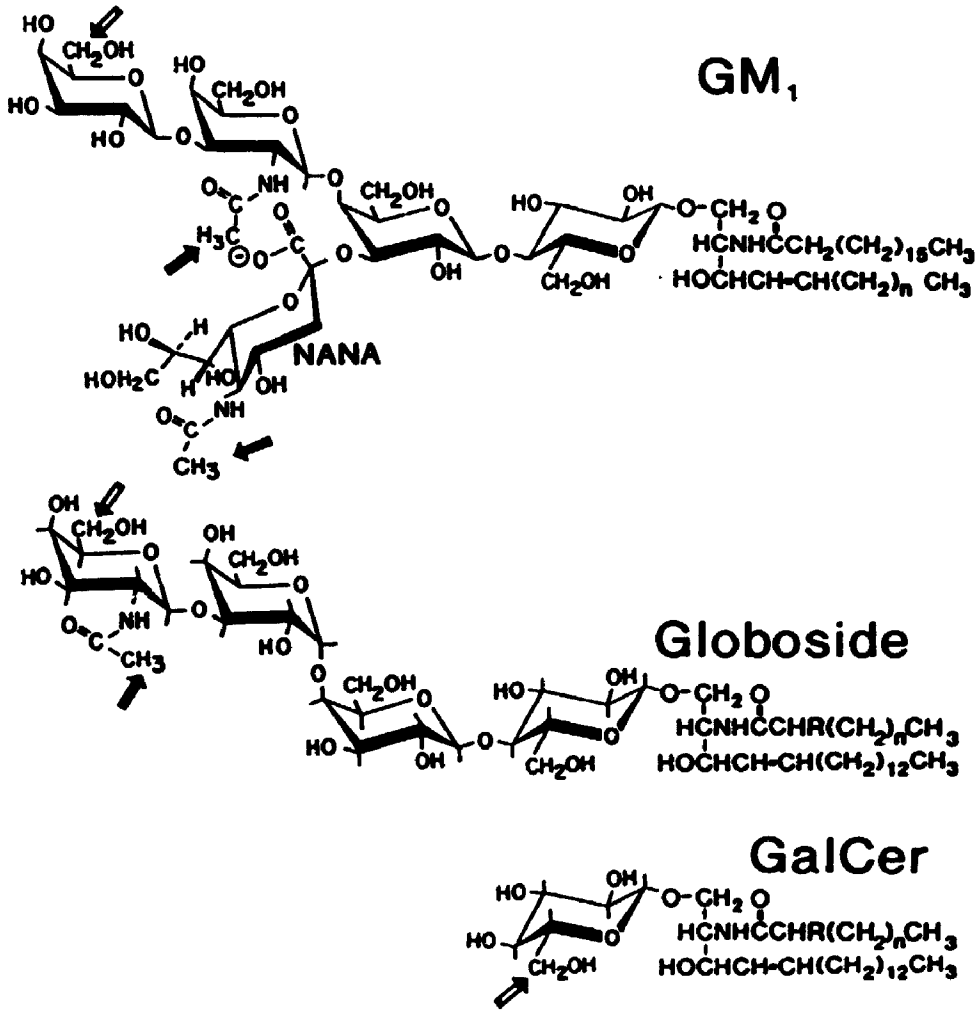
asialo-gm_1.

Globoside from erythrocytes contains the same sugars as asialo-gm_1 but it differs in the types of sugar linkages and the order in which they occur (see Fig. 5.1). Like GalCer, globoside has been held as a classic example of crypticity in the literature. Globoside and its precursors may play a role in kidney and urinary tract infection through their receptor roles for the surface proteins of certain strains of *Escherichia coli* (Kallenius et al., 1981).

Recent analyses, employing two dimensional high resolution NMR in combination with conformational energy calculations, have provided key information regarding headgroup arrangement for intact gangliosides (Acquotti et al., 1990, Scarsdale et al., 1990) and globoside (Scarsdale et al., 1986; Poppe et al., 1990) in polar organic solvent (see also Breg et al., 1989; Maggio et al., 1990 and references therein). The approach has been extended to detergent micelles in the case of globoside (Poppe et al., 1990). Studies of gm_1 (Acquotti et al., 1990) have indicated that there is a relatively well-defined average structure to the carbohydrate portion. Such studies of gm_1, gm_{1b} and gd_{1a} in organic solution have revealed that the position of the NANA residue has an effect on the configuration of the oligosaccharide. This may be less so for globoside (Scarsdale et al., 1986). These studies of pure solvated systems provide important information regarding the behaviour of the oligosaccharide, but correlation with a

Figure 5.1

Structures for the glycosphingolipids, gm_1, globoside, and galactocerebroside (GalCer): arrows indicate deuteron locations. Deuteration of exocyclic hydroxymethyl groups (open arrows) involved the terminal Gal of gm_1, asialo-gm_1 and GalCer, and the terminal GalNAc of globoside. Deuteration of the acetate group (filled arrows) involved NANA and GalNAc residues of gm_1, or GalNAc of globoside and asialo-gm_1. Hydrolytic removal of NANA from gm_1 yielded the asialo species studied. Natural variability in the hydrophobic portions of the molecules is indicated: the sphingosine chain for gm_1 (and asialo-gm_1) is a mixture of C18 and C20 species (n=12 or 14); and the fatty acids for globoside and GalCer are a diverse mixture (R=H or OH, and n=15-23).


GM1
NANA
Globoside
GalCer

membrane context is unknown. ^{2}H-NMR is ideally suited to this task.

The potential of ^{2}H NMR for the study of membrane surface carbohydrate was first demonstrated by Eric Oldfield and colleagues using deuterated monoglycosyl glycosphingolipids (Skarjune and Oldfield, 1979; Skarjune and Oldfield, 1982). More recently, this technique has been applied to several deuterated monoglycosyl- and diglycosyl glycerolipid systems (Jarrell et al., 1986; Jarrell et al., 1987a,Jarrell et al., 1987b; Renou et al., 1989). Limited internal motion of disaccharide chains attached to glycerolipids in fluid bilayer form has been reported (Renou at al., 1989; Adebodun et al., 1992).

Unfortunately, selective deuteration of the moiety of interest is a major stumbling block for the study of the more complex glycolipid species. In the present work, two methods of labelling complex GSLs with deuterium were used. The first employed galactose oxidase as a means of introducing deuterium to the C_6 hydroxymethyl function of galactose and N-acetylgalactosamine. The second technique involved the substitution of the acetate groups of N-acetylgalactosamine (GalNAc) and acetyl neuraminic acid (NANA) residues with deuterated acetate. Gm_1, asialo-gm_1 and globoside were labelled using both techniques and studied within different model membrane systems. The ceramide portion of the GSLs was left unaltered in both labelling procedures.

Different influences, either hydrophobic or hydrophilic in nature, on the behaviour of the GSL carbohydrates are examined in this Chapter. These included the effects of cholesterol, phospholipid composition, calcium and both transmembrane and surface protein.

5.2 **MATERIALS AND METHODS**

DMPC and POPC were obtained from Avanti Polar Lipids, Al. and used without further purification. Cholesterol was from Sigma. Gm_1 was the kind gift of Fidia Pharmaceuticals, Italy. Globoside was isolated from pig's blood as previously described using ethanolic extraction (Mehlhorn et al., 1988).

The -$COCD_3$ (deutero-acetate) derivatives of complex glycolipids were made by partial synthesis following a method outlined by Higashi and Basu for incorporating ^{14}C radiotracers into amino sugars (Higashi & Basu, 1982) which resulted in the retention of the natural fatty acid composition. Briefly, the respective dry glycolipids were dissolved in anhydrous hydrazine, sealed in glass ampoules, and heated at 105°C for 8 hours. The degree of hydrazinolysis was followed by silicic acid thin layer chromatography (TLC), eluting with 55:45:10 $CHCl_3/CH_3OH$/0.2% $CaCl_2$. Hydrazinolysates were dried down and left under vacuum for 6 hours. They were subsequently dissolved in methanol with warming and the addition of benzene, and then

reacetylated using d_6-acetic anhydride (MSD isotopes) in benzene. This material was purified on an Iatrobead (Iatron Laboratories Inc.) column eluted with linear gradients of methanol and water in chloroform/methanol/water (Momoi et al., 1976), and subsequently identified by TLC. Gm_1 has two possible sites for acetate replacement - in the NANA and GalNAc residues. The degree of acetate substitution was found to differ between the two positions as revealed by analysis with proton NMR, following the spectral assignment outlined by Koerner et al. (Koerner et al., 1983). NANA and GalNAc residues were approximately 60% and 28% deuterated respectively.

Asialo-gm_1 was generated by hydrolytic removal of the NANA residue from acetate-deuterated gm_1 using formic acid (Mansson, S. (1973)). The reaction mixture was subsequently dialysed against distilled water at 4° C with frequent changes of the water. Dialysed material was lyophilized and purified on a silicic acid column eluted with a $CH_3OH/CHCl_3$ gradient. The product was identified by TLC.

Deuterium labelling in the exocyclic hydroxymethyl group of GSL oligosaccharide terminal Gal or GalNAc residues was performed using galactose oxidase and $NaBD_4$ as previously described in Chapter 4.

Lipid samples were multilamellar vesicles (MLV) prepared by vortexing above the host transition temperature. Hydration was with buffer containing

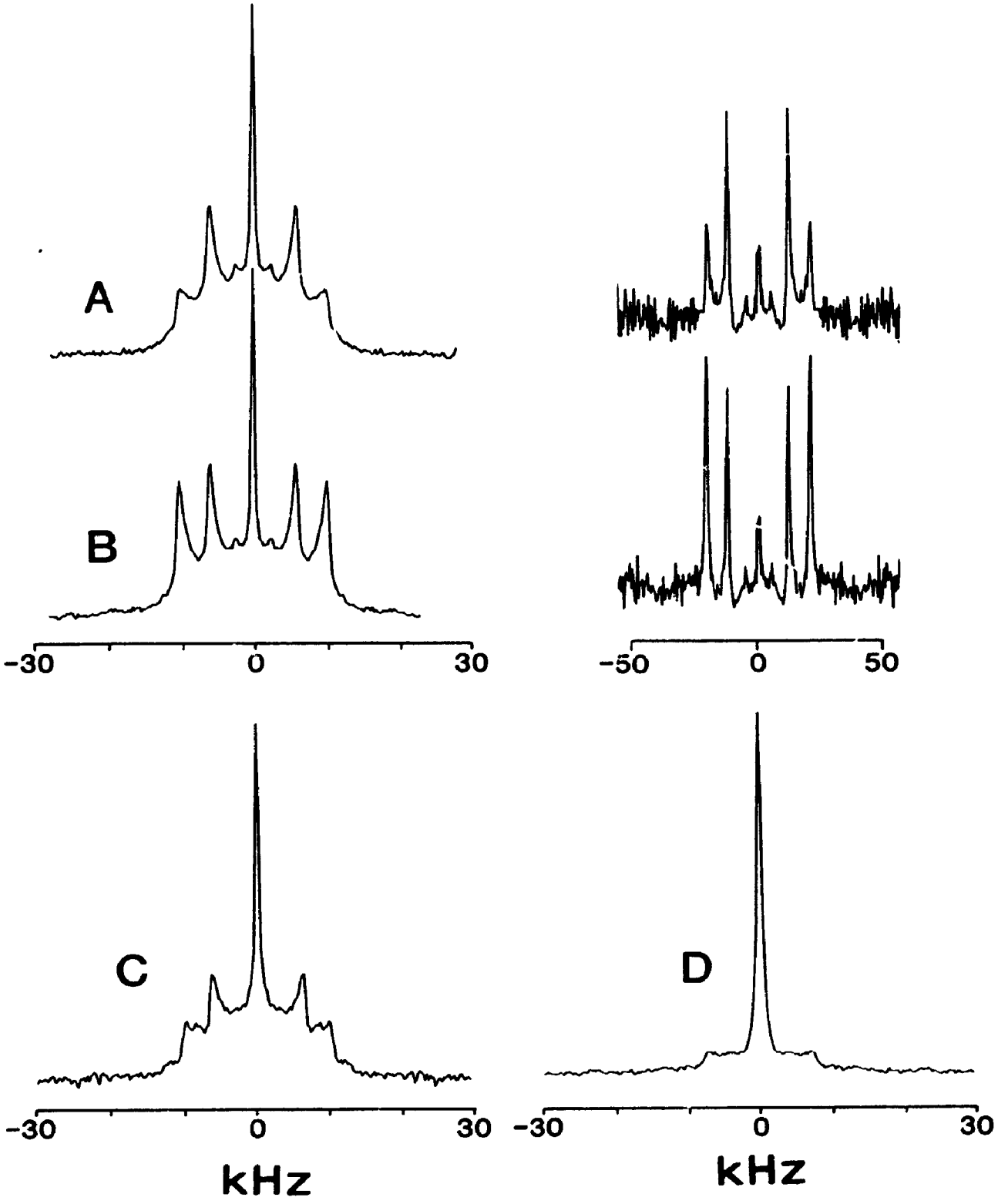
10mM phosphate (pH 7.4) or 5mM Hepes (pH 7.4), with added salts and chelating agents as described in the text and Table captions. Following repeated lyophilization from deuterium-depleted water, samples were rehydrated with deuterium depleted water and freeze-thawed repeatedly. Binary lipid mixtures contained 10 mol% glycolipid while ternary mixtures contained 7.7 mol% labelled lipid and 22.9 mol% cholesterol. The amount of labelled lipid used in each sample varied from 5μM to 16 μM. Sample volumes were 400-700 μl. Wideline ^{2}H-NMR spectra were acquired at 76.6 MHz on a Varian Unity spectrometer with a Doty 5mm solenoid probe. Other details of NMR instrumentation and data handling have been described elsewhere (Chapters 1 and 2). Spectra were acquired from high to low temperature, after preequilibration in the spectrometer well above the host matrix gel-fluid transition temperature.

5.3 RESULTS

Structures of the deuterated glycosphingolipids (GSLs) studied in the present work are illustrated in Figure 5.1. In one group of samples, deuterons were incorporated into the exocyclic hydroxymethyl groups of terminal sugars: yielding -CDHOH (hydroxymethyl label) on the terminal GalNAc

Figure 5.2

Selected ^2H NMR powder spectra for glycolipids deuterated in sugar exocyclic hydroxymethyl groups to give -CDHOH: [αGal]gm_1 (A,B), [αGal]asialo-gm_1 (C), and [αGalNAc]globoside (D). The gm_1 in B was subjected to a second round of oxidation and reduction such that both protons of $-CH_2OH$ were replaced with deuterium. DePaked spectra are included to the right of A and B. In each case the glycolipid was dispersed at 10 mol% in unsonicated POPC bilayers at 40° C. Buffer details are given in the Table captions. Typically 140,000-200,000 transients were averaged.


A
B
C
D
-30
0
30
-50
0
50
-30
0
30
-30
0
30
kHz
kHz

residue of globoside; or on the terminal Gal residue of gm_1, asialo-gm_1, and GalCer. In the other, N-acetate groups of all amino sugars were replaced with -$COCD_3$ (deuteroacetate): this involved the globoside terminal GalNAc residue, the non-terminal GalNAc of gm_1 and asialo-gm_1, and the NANA residue of gm_1.

5.3.1 *General features observed in spectra of GSLs deuterated in specific sugar exocyclic hydroxymethyl groups*

Figure 5.2 presents selected ^2H NMR powder and dePaked spectra for [αGal]gm_1 (5.2A,B), and powder spectra for [αGal]asialo-gm_1 (5.2C) and [αGalNAc]globoside (5.2D) at 40° C. In each case the glycolipids contained deuterium as a -CDHOH group on the fourth (terminal) carbohydrate residue from the membrane surface. At this temperature the basic feature of each powder spectrum was a pair of Pake doublets. A comprehensive set of measured quadrupole splittings ($\Delta\nu_Q$) and, where appropriate, outer/inner splitting ratios corresponding to these and related samples studied, are listed in Table 5.1. The splittings were comparable in magnitude to those observed for similarly labelled Gal residues in the mono- and diglycosyl glycolipids that have been studied previously, for which S_{mol} values of 0.3-0.5 have been recorded (Chapter 4, Skarjune & Oldfield, 1979; Renou et al., 1989). The central sharp peak is a common spectral feature of deuterated lipids in membranes - representing contributions from membranes with high

curvature, and traces of residual HOD. Any population of deuterated carbohydrate residues which are undergoing rapid isotropic motion on the NMR timescale would also give rise to a sharp central peak. However, given the long timescale for the NMR measurements involved (10^{-3}-10^{-5} s), the latter possibility seems likely only if resulting from a subpopulation of highly curved vesicles. The spectral effect of high vesicle curvature has been well described for phosphatidylcholine with deuterated choline methyl groups in fluid bilayers by Curatolo and Neuringer (1986).

All spectra obtained for the lipids investigated here with deuterium in the -CH_2OH group, were similar to ones originally described by Skarjune and Oldfield (1979) for bilayers of the pure monoglycosyl species, [*α*Gal]GalCer (with 16:0 fatty acid) in fluid bilayer form and to those in Chapter 4. These workers determined that the two Pake doublets seen were of equal intensity, and demonstrated that they were readily accounted for by spectral inequivalence of the *pro-R* and *pro-S* deuterons in the -CDHOH group. They pointed out that the spectra could be explained in terms of slow rotation about the C-CDHOH bond, resulting from hydrogen bonding involving -CDHOH. However, they went on to note that subsequent measurements indicate rapid rotation of this group attached to a galactose ring (Skarjune and Oldfield, 1982). Hence it has generally been considered that the equal intensity Pake doublets seen reflect the different average

Tables 5.1 and 5.2

Temperature and acyl chain dependencies of quadrupolar splittings ($\Delta\nu_Q$) for deuterated gm_1, asialo-gm_1, globoside, and GalCer (all with complete natural fatty acid composition except where indicated otherwise) - dispersed in fluid bilayers of POPC, POPC/cholesterol, or DMPC, and buffered at pH 7.4. Two types of sugar deuteration sitesare represented (Figure 5.1): -CDHOH (Table 5.1) and -$COCD_3$ (Table 5.2). Unless stated otherwise, cholesterol concentration was 23 mol% in samples with cholesterol.

† Oligosaccharide motion asymmetric (host T_1 23°C).

* Doubly deuterated in hydroxymethyl function.

‡ This sample was 70:23:7:20 mol ratio POPC/cholesterol/gm_1/GalCer sulphate (ie. a typical POPC/cholesterol/gm_1 sample to which was added a large amount of negatively charged lipid to see the effect of charge close to the surface).

§ These samples contained protein (40 wt% glycophorin or 50 wt% BSA), introduced by exhaustive dialysis (Ketis et al., 1980) of detergent solutions at 4°C (octyl glucoside, 10:1 mol ratio detergent/lipid).

[1] 20 mM NaCl, 5 mM $CaCl_2$, 5 mM HEPES pH 7.4

[2] 100 mM NaCl, 5 mM EGTA, 5 mM HEPES pH 7.4

[3] 10 mM PO_4 pH 7.4

[4]100 mM NaCl, 20 mM $CaCl_2$, 5 mM HEPES pH 7.4

Outer/inner splitting ratios were not calculated for hydroxymethyl-deuterated globoside due to excessive sensitivity to measurement uncertainty in the small central splitting.

Table 5.1 Hydroxymethyl ^2H Data

-CDHOH labelled GSL and Host Matrix	Temp. (°C)	Quadrupole Splitting (± 0.5-1 kHz)	Outer/ Inner ratio (± 0.05-0.1)
GM_1 in POPC[2] (values in brackets are with cholesterol)	40	21.0,12.4	1.7
		21.0,12.4*	1.7
		(21.3,12.2)	(1.7)
	65	17.6,11.4	1.5
		17.6,11.5*	1.5
		(17.1,10.8)	(1.6)
GM_1 in POPC[4] 20 mM Ca^{2+}	40	21.5,11.9	1.8
	65	19.1,11.5	1.7
§GM_1 in POPC/cholesterol[2] with Glycophorin	40	21.0,11.8*	1.8
‡GM_1 in POPC[3] with cerebroside SO_4	40	22.5,12.9	1.7
Asialo-GM_1 in POPC[2] (values in brackets are with cholesterol)	40	20.5,13.2	1.6
		(19.9,12.3)	(1.6)
	65	16.6,11.4	1.4
		(16.1,10.8)	(1.5)
Globoside in POPC[3]	40	15.3,1	
Globoside (non-OH FA) in POPC[3]	40	15.1,1	
		(16.0,1)	
Globoside (-OH FA) in POPC[3]	40	16.2,1	
		(16.7,1)	
Globoside (non-OH FA) in DMPC[3]	15	†	
	25	17.8,1	
GalCer in POPC[3]	40	18.8,10.5	1.8
18:0 FA	40	17.7,9.7	1.8

Table 5.2 Acetate ^2H Data

-$COCD_3$ labelled GSL and Host Matrix	Temp. (°C)	Quadrupole Splitting (± 0.5-1 kHz)
GM_1 in POPC[2] (values in brackets are with cholesterol)	15 25 35 40 65	10.7 10.5(10.1) 9.7(9.7) 9.3 7.8
GM_1 in POPC[4] 20 mM Ca^{2+}	15 40 65	11.0 9.9 8.2
2 mM Ca^{2+}	23	10.5
Asialo-GM_1 in POPC[2]	40	9.6
Globoside in POPC[3] (values in brackets are with cholesterol)	15 25 40 65	1(1) 1(1) 1(1) (<1)
18:0 FA	25 35	1.6(1.6) 1 (1.6)
Globoside (non-OH FA) in POPC[3]	40	1.6(1.6)
Globoside (-OH FA) in POPC[3]	40	1.6(1.6)
Globoside in DMPC[3]	15	†
§Globoside in POPC/cholesterol[1] with Glycophorin	25	(1.5)
§Globoside in POPC[1] with BSA, 2 mM Ca^{2+}	23	1

spatial orientations for the two stereoisomeric deuterons as they undergo rapid interconversion amongst rotamers (gauche(+), gauche(-) and trans) about the C-CDHOH bond (Renou et al., 1989; Chapter 4).

Direct assignment of these spectral splittings to specific (*pro-R* or *pro-S*) deuterons has not been reported for glycolipids. However, the behaviour of the -CDHOH group has been considered in detail by Renou et al. (1989) for the galactose residue of the glycero-glycolipid, lactosyl-1,2-di-O-tetradecyl-*sn*-glycerol. The latter species also gave a pair of Pake doublets of equal intensity when studied in fluid bilayer form: assignment of the inner splitting to the *pro-R* position was consistent with independently-derived orientation of the Gal residue, presuming rapid reorientation about the C-CDHOH bond. We demonstrated (below) that spectral interpretation in terms of rapid -CDHOH reorientation for the Gal residue also seemed likely for the complex oligosaccharide chain of gm_1.

Figure 5.2A shows a typical powder spectrum of gm_1 labelled in the $-CH_2OH$ group of the Gal residue *via* a single exposure to galactose oxidase followed by $NaBD_4$ reduction. The intensities of the Pake doublets are not equal (more clearly seen in the dePaked spectrum to the right of the powder spectrum). In contrast, the correspondingly deuterated Gal residue of GalCer (Skarjune & Oldfield, 1979; see also Chapter 4) and lactosyl-1,2-di-O-tetradecyl-*sn*-glycerol (Renou et al., 1989) produced paired Pake doublets

of equal intensity (see also Figure 5.4E below). Either of two possibilities could explain this observation: i) the -CDHOH group in gm_1 is rotating slowly on the NMR timescale amongst 2 or more conformers whose corresponding spectral peaks happen to be hidden under one another, and/or ii) there is unequal deuteration of the (two) spectrally inequivalent positions on the carbon.

The first of these possibilities presumes that, in contrast to literature expectations for galactose (Hayes et al., 1982), and contrary to previous ^2H NMR observations on Gal residues (Skarjune & Oldfield, 1979; Renou et al., 1989), rotation of -CDHOH in the gm_1 terminal Gal residue is not rapid - as a result of the additional sugars present in the molecule or different interactions with the surface environment for the more complex GSL. If two or more rotamers associated with the C-CDHOH bond were in slow exchange as has been reported to be the case for this group in glucose residues of several simple glycolipids (Jarrell et al., 1986; Jarrell et al., 1987a,b; Renou et al., 1989), the spectral intensities would be functions of the relative rotamer populations. This possibility would require that fortuitous peak overlap occur, resulting in merging of at least 8 spectral peaks to the 4 actually observed, with the additional coincidence that the ratio of inner-to-outer peak intensity fits the pattern described below following a second round of oxidation/reduction.

The second possibility may be understood in terms of the reaction stereochemistry, as follows. The process used in the present work for introduction of ^2H nuclei into gm_1 entails enzymatic oxidation of the $-CH_2OH$ on the terminal Gal moiety to produce an aldehyde. The subsequent step is reduction of the aldehyde with $NaBD_4$. This process would be expected to result in the introduction of equal amounts of *pro-R* and *pro-S* deuterons if there were no stereo-selectivity involved in the reactions. If, however, there is selectivity, the relative amounts of the isomers would differ and could be a source of the observed inequality of their peak intensities. The stereoselectivity of this process has been considered in detail by Maradufu et al. (1971) for the monosaccharides, galactose and β-methyl galactoside, dissolved in water. In work with lipids, these reactions are carried out in 1:1 THF/water to improve solubility and presumably involve large micelles for the neutral lipids.

Maradufu et al. (1971) indicated that the initial oxidation of free galactose in aqueous solution may be expected to specifically remove the *pro-S* hydrogen, producing a (symmetric) non-deuterated aldehyde. Subsequent reduction of this compound with $NaBD_4$ leads preferentially to the *pro-R* (mono)deuterated product in a 3.6:1 ratio: ie. after an initial oxidation/reduction process, the *pro-R* (mono)deuterated Gal should be the predominant product, with considerably less *pro-S*. Gm_1 is the only water

soluble glycolipid studied in the present work. Hence the fact that the gm_1 spectrum in Figure 5.2A displays an inner/outer intensity ratio of about 2:1 might suggest that the inner Pake doublet is associated with the *pro-R* deuteron and the outer doublet with the *pro-S*. According to Maradufu et al., ^1H nuclei at the *pro-S* position should be selectively removed 7.7 times more rapidly than ^2H nuclei at the same location due to a kinetic isotope effect. They also found that oxidative removal of the nucleus at the *pro-R* position does not occur, as mentioned above. Hence, under our reaction conditions, *pro-S* ^2H nuclei may be expected to be unaffected by a second round of enzymatic oxidation. Neither ^1H nuclei nor ^2H nuclei at the *pro-R* position should be affected. As a result, subsequent reduction with $NaBD_4$ should produce essentially complete deuteration of the $-CH_2OH$ group, with the exception of the residual small amount of *pro-R* left undeuterated after the first round of reaction. If one may assume that the resultant $-CD_2OH$ group is undergoing rapid rotation such that each Pake doublet corresponds to either a *pro-S* or *pro-R* deuteron, the stereoselectivity described would be expected to produce almost equal intensity for the two Pake doublets, but with a slight preponderance of that associated with the *pro-S* position - in agreement with the observation of reversal of the inner/outer intensity ratio in Figure 5.2B. The results are highly consistent with the inner Pake doublet being associated with the *pro-R* deuteron, and rapid rotation of the

hydroxymethyl group.

It is difficult to accurately judge relative peak intensities for [αGalNAc]globoside since, with the natural fatty acid mixture, the inner Pake doublet is not distinctly resolved from the central peak (5.2D)(see below). However, the basic features are readily understood in terms of the logic described for gm_1 and simpler glycolipids.

5.3.2 *General features observed in spectra of GSLs deuterated in amino-sugar acetate groups*

For (symmetrically rotating) deuterated methyl groups, it is convenient to consider a 'resultant' C-D bond vector directed along the C-CD_3 bond attaching the methyl group: this can be dealt with in equation (3) by considering Θ to be the angle between the C-CD_3 vector and the molecular long axis, and introducing an additional factor of 1/3. Independent motion of the rigid, planar, -$NHCOCD_3$ group about the C-N bond linking it to the sugar ring is highly restricted (Yadav & Luger, 1980; Acquotti et al., 1990; Poppe et al., 1990) and the C_2-N bond is directed parallel to the C-CD_3 bond; so that libration about C_2-N does not importantly alter the orientation of the C-CD_3 vector relative to the sugar ring. Moreover, the effect of rapid rotation parallel to this axis (ie. about the C-CD_3 bond) is already explicit in the calculation used when a factor of 1/3 is introduced to

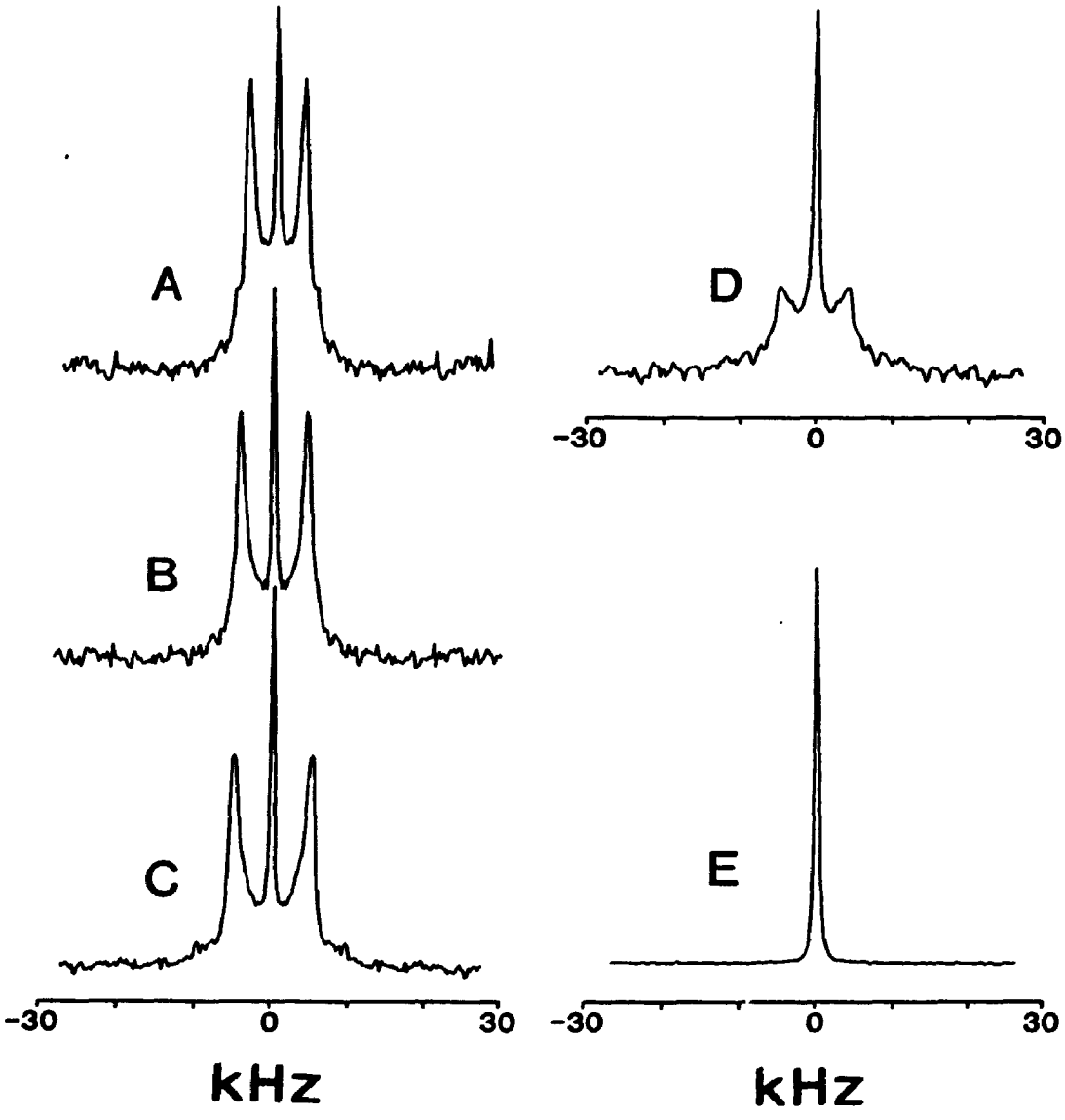
equation 1) as described above. Hence, a given amino-sugar deuterated in this fashion and associated with a glycolipid in a fluid membrane, should produce one Pake doublet. The existence of different glycolipid populations long lived on the NMR timescale, would be expected to produce multiple Pake doublets.

Figure 5.3 presents typical ^2H NMR spectra for [d_3NANA d_3GalNAc]gm_1 (5.3A-C), [d_3GalNAc]asialo-gm_1 (5.3D), and [d_3GalNAc]globoside (5.3E), dispersed as minor components in bilayers of POPC. Each spectrum was characterised by a predominant single Pake doublet - although in the case of the globoside with natural fatty acids (5.3E) the small doublet splitting was poorly resolved from the central peak (see below). For asialo-gm_1 and globoside, only one Pake doublet was expected per glycolipid average orientation and environment, as there was only one amino sugar. For gm_1, high resolution analysis indicated significant deuteration at the NANA and GalNAc positions (3:1 ratio); so that two doublets were expected in the wideline study. It would appear that the quadrupole splittings of these doublets were similar, and that as a result the GalNAc doublet was not well resolved (although small shoulders exist in 5.3A,B).

The maximum splitting that might be observed for a rapidly rotating - CD_3 group on a lipid in fluid membranes is about 42 kHz. This would be the

Figure 5.3

Selected ^2H NMR powder spectra for glycolipids deuterated in amino-sugar acetate groups to give -$COCD_3$: [d_3NANA d_3GalNAc]gm_1 at 65°C (A), 40°C (B) and 25°C (C); [d_3GalNAc]asialo-gm_1 at 40°C (D) and [d_3GalNAc]globoside at 40°C (E). All samples contain GSL dispersed at 10 mol% in POPC bilayers. Buffer details are given in the Table captions. Typically 60,000 transients were averaged.


A
B
C
D
E
-30
0
30
-30
0
30
kHz
kHz

case if S_{mol} were 1.0 and the methyl group were oriented perpendicular to the plane of the membrane. Hence, the 10 kHz splittings seen for gm_1 indicate considerable preservation of orientational order. In the case of globoside, the splittings were in the 1 kHz range and might suggest just the opposite - ie. a small value for S_{mol} (Table 5.1). However, it was clear that for globoside with a -CDHOH group in the terminal sugar residue (Table 5.1 and Figure 5.2D), the spectra displayed outer splittings of magnitude comparable to those seen for gm_1 labelled in the same fashion (eg. 15-16 kHz *vs* 21 kHz at 40°C). Given the existence of three significant rotamer populations for a Gal -CDHOH group calculated by Renou et al. (1989), the maximum splitting that might be observed for such a deuteron was probably in the range of 60 kHz, assuming no independent wobble of the labelled sugar. Hence S_{mol} for the globoside chain cannot be close to 0.0, and one may rule out the suggestion that the small quadrupole splittings obtained for acetate-labelled globoside result solely from very high disorder of the oligosaccharide chain.

5.3.3 *Effect of GSL hydrophobic portion*

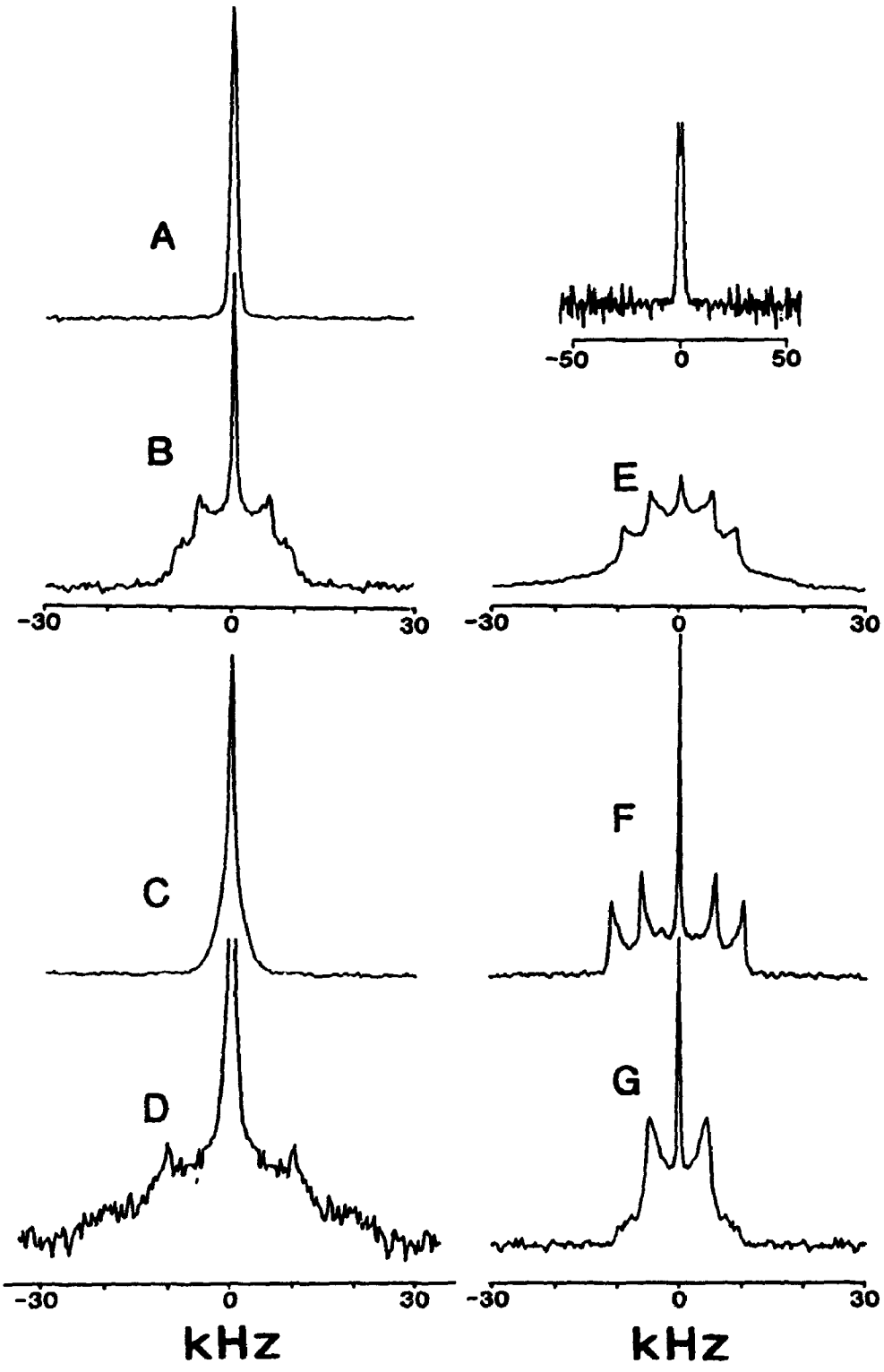
All GSLs studied in the present work were homogeneous in having 90-96% sphingosine base (dihydrosphingosine comprising the remainder)(Abrahamsson et al., 1972; Thompson & Tillack, 1985). The

natural fatty acid composition of gm_1 from the source studied is primarily 18:0 (Corti & Degiorgio, 1980; Sonnino et al., 1985). However, in gm_1 the sphingosine chain length is divided between two families: 60-70% is C18 and 30-40% is C20 (Corti & Degiorgio, 1980; Sonnino et al., 1985) - as distinct from the situation for globoside and GalCer which are almost totally C18 sphingosine (Sweeley, 1989). Thus, while the gm_1 studied had homogeneous acyl chain length, its alkyl chain length was divided between two large fractions. Examination of the hydroxymethyl-deuterated [dGal]gm_1 examples in Figure 5.2A,B shows that, apart from the sharp central component, well over 90% of the spectral intensity is localised to the single, fairly homogeneous, two-doublet spectrum already described for a -CDHOH group in one average environment. Similarly, in the spectra of [d_3NANA d_3GalNAc]gm_1 in Figure 5.3A-C, greater than 90% of the signal intensity can be accounted for by invoking a single population of molecules. Clearly the two-carbon length difference in the alkyl chain can have very little effect on headgroup behaviour in the systems represented in Figures 5.2,5.3. Similar results were obtained for asialo-gm_1 (Figure 5.2C,5.3D).

The fatty acids of globoside from pig erythrocytes are highly heterogeneous (some 90% are C20 or longer and just over half are α-OH)(Hanahan et al., 1971; Bunow & Levin, 1980). They may be separated on silicic acid into faster and slower running fractions enriched in non-

Figure 5.4

^{2}H NMR powder spectra for: [d_3GalNAc]globoside at 40°C in POPC/cholesterol (A) - depaked spectrum to right; [dGal]asialo-gm$_1$ at 40°C in POPC/cholesterol (B); [d_3GalNAc]globoside at 15°C in DMPC (phase transition temperature 23°C, compare to fluid matrix in Figure 5.3E)(C); [dGalNAc]globoside at 15°C in DMPC (phase transition temperature 23°C, compare to fluid matrix in Figure 5.2D)(D); [dGal]GalCer with natural fatty acid mixture at 40°C in POPC (E); [dGal]gm$_1$ (two cycles of oxidation/reduction) at 40°C in POPC with 20 mM Ca^{2+} (F); [d_3NANA d_3GalNAc]gm$_1$ at 40°C in POPC with 20 mM Ca^{2+} (G).


A
B
C
D
E
F
G
-50
0
50
-30
0
30
-30
0
30
-30
0
30
-30
0
30
kHz
kHz

hydroxy fatty acids and more polar α-OH species respectively. Chain length and unsaturation are reportedly similar for the non-hydroxy and α-OH species (Hanahan et al., 1971). A typical spectrum of natural globoside deuterated with the sensitive -CDHOH probe ([*d*GalNAc]globoside) is shown in Figure 5.2D. It displays an outer Pake doublet with large quadrupole splitting, and an inner Pake doublet whose splitting is poorly resolved from the central peak. These doublets were more distinctly resolved in separate experiments with the faster and slower running (TLC) fractions; but the largest differences (in the outer splitting) were close to experimental error (Table 5.1). Thus, although spectral differences amongst the subpopulations may be significant, the variation actually measured in the current experiments can be accounted for by modest alterations in S_{mol} and/or orientation of the carbohydrate portion. The same was true in the case of the $-CD_3$ labelled [d_3GalNAc]globoside, (Figure 5.3E). The one acetate label per globoside headgroup should result in a single Pake doublet. This was seen for globoside with only a C-18 fatty acid, however the splitting was barely resolvable (Barber et al., 1994). If globoside fatty acid nature determined headgroup conformation or order, we would expect the spectrum in Figure 5.3E to show coexisting pairs of Pake doublets, reflecting the wide range of natural fatty acids present. Instead, the spectral lines were simply broadened. Powder spectra of the faster and slower running fractions

displayed single Pake doublets with very small splittings (Table 5.2).

Further insight into this phenomenon was obtained from the monoglycosyl species, [αGal]GalCer having natural fatty acid composition. A spectrum of this species in POPC is included as Figure 5.4E. It retained the features demonstrated originally for fluid bilayers of pure [αGal]GalCer with only 16:0 fatty acid (Skarjune & Oldfield, 1979). The natural fatty acids of GalCer from beef brain comprise many α-OH species, and significant amounts of material with both C18 and longer acyl chains (>75% of the fatty acids are C20 or longer, and roughly half are α-OH fatty acids (Bunow & Levin, 1980)). The spectrum in Figure 5.4E demonstrated quadrupole splittings of 10.5 and 18.8 kHz at 40°C. These values were within experimental error of those previously recorded for [αGal]GalCer with single pure fatty acids (18:0, 18:1, 24:0, 18:0 α-OH) in fluid POPC bilayers (Chapter 4). They were also within experimental error of the major splittings measured for the natural α-OH and non-hydroxy fractions which had been separately isolated prior to enzymatic oxidation and subsequent reduction with $NaBD_4$ in the present work (Table 5.1).

A variety of influences were subsequently imposed on the membranes studied, in an attempt to identify factors that might directly alter orientation and behaviour of the recognition sites, or modify their susceptibility to elements described above.

5.3.4 *Effect of cholesterol*

Tables 5.1,5.2 provide data for samples similar to those containing the complex GSLs described above, but with cholesterol in the membrane. In general, 23-30% cholesterol did not change the quadrupole splittings within experimental error in the fluid membranes studied. Examples of the spectra obtained are presented for -$COCD_3$ labelled globoside (5.4A, depaked version included) and -CDHOH labelled asialo-gm_1 (5.4B).

5.3.5 *Effect of temperature/membrane fluidity*

Only very modest temperature variation was apparent in spectra of glycolipids studied in fluid membranes (eg. Figures 5.3A-C and Tables 5.1,5.2). Alteration of the host matrix to a gel phase had however produced dramatic spectral changes. This is apparent in Figure 5.4C,D which display spectra for [α_3GalNAc]globoside (5.4C, compare to 5.3E) and [αGalNAc]globoside (5.4D, compare to 5.2D) with natural fatty acid composition at 10 mol% in DMPC below the 23°C phase transition of the host matrix. It was clear that, in a gel phase host matrix, the spectrum changed drastically to reflect altered (slowed) motional properties. These results in DMPC at 15°C contrasted with spectra obtained for globoside with natural fatty acid composition in POPC, which retained the (fluid phase)

features already discussed to well below 10°C.

5.3.6 *Effect of desialation*

Under a given set of conditions, there was striking quantitative similarity between spectra for [αGal]asialo-gm_1 (5.2C) and those corresponding to the identically deuterated [αGal]gm_1 from which it was derived (5.2A). This was also true for the -$COCD_3$ labelled species (Figure 5.3). The -$COCD_3$ spectra shown for gm_1 in Figure 5.3A-C should display one Pake doublet for the NANA residue and a second doublet associated with deuterons on the GalNAc residue (Figure 5.1). Based upon ^1H NMR characterisation (not shown) of the product of hydrazinolysis in the present work, the GalNAc Pake doublet was expected to be 1/3 the intensity of that associated with the NANA residue. A second Pake doublet was not obviously present, in this or in samples more extensively hydrazinolysed prior to reacetylation (although a shoulder can be seen at the high temperature). It appeared that the peaks associated with -$COCD_3$ on the NANA of gm_1 closely overlapped those associated with -$COCD_3$ on the GalNAc residue. Indeed, selective removal of the α_3NANA residue from gm_1, so that only the GalNAc deuterons remain, left much the same spectrum: a splitting of 9.6 kHz for asialo-gm_1 at 40° compared to a value of 9.3 kHz for gm_1 (Figure 5.3D and 5.3B respectively, Table 5.2). Note too that introduction of an

additional negative charge near to the membrane surface also had little effect: when sulphatide (GalCer sulphate) was incorporated at 20 mol% into POPC/cholesterol membranes containing gm_1 the alteration in the spectrum was close to experimental uncertainty (Table 5.2).

5.3.7 *Other factors*

A subset of the systems dealt with above were further examined in the presence of ionic calcium - generally at the relatively high concentration of 20 mM. Figure 5.4F shows the spectrum of -CDHOH labelled gm_1 in the presence of 20 mM Ca^{2+}, and Figure 5.4G shows that for -$COCD_3$ labelled gm_1 in the presence of the same high Ca^{2+} concentration. The features already described remain, and the quadrupole splittings were similar to values without divalent cation (Tables 5.1,5.2). In general it was observed that addition of 20 mM Ca^{2+} could induce some broadening of the original features with minimal effect on the measured splittings. Ca^{2+} was also seen to induce generalised and reversible broadening of the spectral features of asialo-gm_1, while the 90° edges remained close to the values measured without the divalent cation (Tables 5.1,5.2).

High concentrations of the integral glycoprotein, glycophorin, were incorporated into POPC/cholesterol liposomes containing gm_1 deuterated in the terminal Gal -CH_2OH group or in the amino-sugar -$COCH_3$ groups. The

spectral splittings were unchanged within experimental error at 15°C and 25°C (Table 5.1, spectra not shown). Similarly, neither glycophorin nor a very high surface concentration of serum albumin had appreciable effect on [d_3GalNAc]globoside spectral characteristics (Tables 5.1,5.2).

5.4 DISCUSSION

In Chapter 4 the simple GSL, GalCer, demonstrated a preferred average oligosaccharide orientation that was virtually independent of its fatty acid characteristic. The same approach to deuteration introduced in Chapter 4, was extended to complex glycolipids. Spectral interpretations demonstrated previously for glycerolipids (Renou et al., 1989) - were shown to be appropriate for the more complex chain of gm_1. Spectral inequivalence of the two deuteron locations in -CDHOH provides considerable potential for sensitivity to alterations in headgroup orientation and behaviour. Unfortunately, the existence of incompletely-characterised rotational conformers about the C-C bond attaching the group to the sugar ring adds a difficult-to-handle factor for considerations of carbohydrate headgroup orientation. Also, the fact that separate spectral peaks are observed for each deuteron minimizes the signal intensity when this approach is used (although if the oxidation/reduction cycle is repeated, it can be improved by more fully labelling both sites). Another possible source

of concern when using only this (enzymatic) labelling technique is that, theoretically, certain subpopulations of a given GSL could be preferentially labelled.

The second technique for probe introduction employed in the present work involved deuteration of acetate groups on amino-sugars. The hydrazinolysis method used here led to substitution of each acetate, and did not cleave the amide linkage attaching the (single) fatty acid to the GSL ceramide backbone (presumably because of its sequestration within some form of lipid aggregate (Higashi & Basu, 1982)). -$COCD_3$ groups gave a 3-fold improvement in signal-to-noise ratio as a result of spectral equivalence of the 3 deuterons, and made possible more direct correlation with spatial orientation of the carbohydrate portion.

Wideline ^2H NMR is a non-perturbing and sensitive method of probing spatial orientation and degree of motional order within deuterated molecules. Thus it was noteworthy that each of the oligosaccharide recognition sites studied, dispersed as a minor component at the surfaces of fluid membranes, was seen to have a preferred average conformation. Perhaps more remarkable, was preservation of these average conformations in the face of alterations in hydrophobic structure, temperature, and surroundings. One might suggest that this resulted from the fact that the carbohydrate chains and/or probe sites chosen, were extremely disordered

and flexible such that their motions were insensitive to local occurrences. However this was not the case. The maximum 90°-edge spectral splitting anticipated for a rapidly rotating -CD_3 group attached to a molecule which was itself undergoing rapid rotation about a molecular axis, was 42 kHz; and for a -CDHOH group it was likely in the neighbourhood of 60 kHz (depending on the number and statistical likelihood of conformers about C-CDHOH). Hence the quadrupole splittings observed in the present work suggested that values of S_{mol} for the deuterated segments of gm_1, asialo-gm_1, and globoside, could not have been less than 0.25-0.5. Interestingly, S_{mol} values reported for β-linked mono-glycosyl species fall within this range (Skarjune & Oldfield, 1982; Jarrell et al., 1986; Jarrell et al., 1987a,b). Independent wobble of individual sugar residues in the chain must be limited. Thus the approach used here was adequate to detect phenomena affecting conformation and order of the GSL oligosaccharide chains. The possibility that more significant headgroup alterations occurred which involved small (undetected) populations of the glycolipids studied cannot be excluded.

As previously mentioned, glycolipid participation in recognition events can be importantly modulated by changes in lipid structure, and by membrane fluidity or cholesterol and protein content. The concept of receptor "crypticity" derives from such observations (Alving et al., 1980; Kannagi et al., 1983; Shichijo & Alving, 1985; Hakomori, 1986; Lampio et al.,

1986; Curatolo and Neuringer, 1986; Mehlhorn et al., 1988; Stromberg et al., 1991; Stewart & Boggs, 1993 ; Kiarash et al., 1994, and references therein). The underlying mechanisms warranted consideration for their involvement in triggering events connected with initiation of specific signalling pathways at cell surfaces. Some such examples of oligosaccharide 'communication' with the hydrophobic region may be dependent on properties of the systems in which the measurement was made - eg. gel phase nature of the bilayer membranes (Hamilton et al., 1994) or the presence of membrane proteins. X-ray studies form an important basis for considering oligosaccharide control by lipid structure, as they suggest effects of GSL backbone on headgroup orientation. For instance, in single crystals of GalCer there is evidence that fatty acid α-hydroxylation could alter carbohydrate conformation *via* H-bonding (Pascher & Sundell, 1977, see also Nyholm et al., 1990). A question that remains is, the extent to which these basic forces identified in single crystals and model systems may be altered for receptors dispersed in (often fluid) membranes (eg. Bunow & Levin, 1980; Curatolo, 1987).

Parameters suggested to be capable of inducing receptor crypticity include lateral distribution, motional properties, orientation, and degree of protrusion from the membrane surface. The technique of ^{2}H NMR, as employed in the present work, has particular application to a subset of

these. The presence of deuterated GSL in a gel phase - a condition such that both lateral and rotational diffusion were strongly reduced - led to a very distinctive spectrum. Thus we should have detected the formation of domains highly enriched in glycolipid, since these would be gel phase at the temperatures studied (Maggio et al., 1985; Bunow & Levin, 1988 and references therein). However, our approach would not be sensitive to minor receptor enrichment leading only to increased local order in fluid membranes, based on our observations of the minor effect of cholesterol. Hence the present experiments said little about the role of moderate changes in GSL lateral distribution in the systems examined. Where briefly tested, host matrix rigidification induced striking spectral alterations - interpretable in terms of loss of rapid symmetrical rotation of the GSL molecule. This likely indicated that the carbohydrate portion was importantly influenced, at least in its axial diffusion, by immobilisation of the membrane-inserted portion. The experiment did not rule out the possibility that GSL clustering in the gel phase, with resultant steric hindrance, contributed to the oligosaccharide motional changes. Altered headgroup order and orientation were sensitively tested for, as was headgroup protrusion if it influences either of these parameters. Lateral diffusion in fluid membranes may be expected to alter NMR relaxation times, but not the spectral parameters measured here.

Chain length and hydroxylation are key factors identified as possible sources of recognition site modulation in studies of cryptic[h] which were examined in Chapter 4 for the simple GSL GalCer. With regard to the present complex GSLs examined, it is known that a sphingosine chain length difference of 2 carbons is sufficient to influence the size of pure ganglioside micelles (Yohe et al., 1976). Thus it is noteworthy that a 2-carbon alteration in the sphingosine chain length of gm_1 and asialo-gm_1 had no obvious spectral effect, as measured by deuterons in the terminal NANA or Gal residues and in the internal GalNAc. The faster and slower running fractions of globoside have also been reported to show differences in micelle size related to fatty acid composition (Tinker et al., 1976). However, our spectra of globoside with a wide variety of natural fatty acids showed evidence of only minor spectral differences in the headgr[illegible]p region. The maximum quantitative change in spectral splittings associated with fatty acid alteration was 1-2 kHz, which is no more than 5-10% of the range over which splittings could have varied for orientational changes between 1 and 90°. These small differences have reflected correspondingly small changes in orientation or S_{mol}. The above findings appear to be generalisations of our earlier observation that ^2H NMR spectra of [*a*Gal]GalCer dispersed in fluid POPC bilayers were the same for 5 different synthetic fatty acids (Chapter 4). As mentioned above, a concern when studying GSLs deuterated *via* the

enzymatic-oxidation/reduction cycle was that subfractions might selectively fail to react. This was probably minimised in the organic solvent mixture used when oxidation times were long (as here). We have not observed failure to react for any of the natural fractions or for more defined fatty acids (Chapter 4, Stewart & Boggs, 1993a, also see Chapter 4). The acetate labelling procedure does not rely on enzymatic oxidation, and gave results consistent with the enzymatic approach.

It was also noteworthy that the effect of temperature on the above observations was very modest, in spite of the fact that sample warming could potentially alter intermolecular associations and collisions as well as the effects of intramolecular forces. The temperatures chosen were influenced to some extent by the fact that, above 40°C, up to 10 mol% in POPC (without cholesterol), GalCer remains dispersed in a fluid matrix. Below 40°C, depending on the GSL fatty acid, there can be measurable separation of a GSL-enriched phase as was noted in Chapter 4. We have not seen evidence of phase separation in POPC near 40°C with gm_1 and globoside - which have considerably lower main transition temperatures than GalCer (Maggio et al., 1985)(unpublished observations). The small temperature-induced changes seen in the current work are inconsistent with any major structural alterations in the carbohydrate headgroup. One form of intermolecular association that has attracted interest with regard to

oligosaccharide chains, is carbohydrate association with the membrane surface. Little is known about the forces governing such interactions (see for instance Stromberg et al., 1991; Aubin & Prestegard, 1993). However, binding of soluble carbohydrates to liposomes is well known; and it has been postulated that effects on water of hydration are involved (Crowe et al., 1987). Hence it is conceivable that long carbohydrate chains could associate with bilayer surfaces. Such associations would probably involve conformational distortion and would be expected to be temperature sensitive. However, it would appear that, in fluid membranes, the effect of increased temperature on the complex GSL oligosaccharide was limited to a modest decrease in order. This result was consistent with the observation of Bechinger et al. (1988) that simple neutral glycolipids induced no measurable alteration in the ^2H NMR spectrum of the choline headgroup of POPC.

Pure POPC bilayers exhibit a main transition temperature of -3°C (Davis & Keough, 1985), and have low chain order at the temperatures investigated. High cholesterol concentration is known to greatly restrict chain flexibility in fluid phospholipid matrices, while having much less effect on rotational (whole body) diffusion (Oldfield & Chapman, 1972; Demel & De Kruyff, 1976; Yeagle, 1985; Vist & Davis, 1990). In addition, cholesterol increases fluid bilayer thickness. One might suggest that the oligosaccharide

of GSLs should be more sensitive to variations in the hydrophobic region in cholesterol-rich membranes, where spatial constraints would be more critical. It has been recently observed that deuterons at C_2 of GalCer fatty acids showed evidence of greater orientational differences between the 18:0 and 24:0 species when the membranes contained cholesterol, particularly at low temperature (Hamilton et al., 1994). Some of the earliest systematic observations concerning glycolipid (and phospholipid) cryptictiy noted important effects of cholesterol (Brulet and McConnell, 1977; Balakrishnan et al, 1982; Utsumi et al, 1984; Stanton et al, 1984; Mehlhorn et al 1988; Stewart and Boggs, 1990). Nevertheless, in the present work the difference between the spectra of the cholesterol containing samples as compared to the same systems without cholesterol was found to be close to or within experimental error. Presumably this was a further reflection of the lack of oligosaccharide sensitivity to even quite large structural and conformational alterations within the membrane-inserted portions of GSLs dispersed in fluid membranes.

Neuraminic acidic (NANA) residues are important features of cell surfaces. They have been implicated in recognition events and in membrane structure. As a result, natural extracellular neuraminidase activity has attracted interest and is suggested to be of significance in some signalling events (reviewed in Sweeley, 1993). Selective removal of NANA

from gm_1 left the uncharged tetrameric chain, Galß1→3GalNAcß1→4Galß1→4Glc (asialo-gm_1). Hence, the design of the current experiments made it possible to test the relationship amongst conformation, behaviour, and the presence of a negatively charged NANA residue in a surface-attached oligosaccharide chain. One might have anticipated a measurable spectral effect of removing NANA from gm_1 since the sugar is attached in the middle of the chain, and is the only source of charge in the molecule. High resolution NMR of gm_1, gm_{1b}, and Gd_{1a} in DMSO/2% H_2O solution have indicated conformational effects related to placement of the NANA residue (Scarsdale et al., 1990). However, under a given set of conditions, there was striking quantitative similarity between spectra for asialo-gm_1 deuterated in the terminal Gal residue ([*d*Gal]asialo-gm_1) and those corresponding to the identically deuterated gm_1 from which it was derived - the spectral differences between the two glycolipids being zero within experimental error. The same result was obtained when asialo-gm_1 was probed with -CD_3 on the next-to-terminal GalNAc ([d_3GalNAc]asialo-gm_1): that the spectral values were within experimental error of those for gm_1 under a given set of conditions. It seemed therefore that gm_1 and asialo-gm_1 have very similar conformations and order parameters at the (fluid) membrane surface. This apparent insensitivity to removal of the charged NANA residue on gm_1 led us to test the opposite experiment - addition of

GalCer SO_4^{2-} to form a layer of negatively charged groups close to the membrane surface. A cholesterol-containing membrane was chosen to minimise the anticipated 'stiffening' effect of added (high-melting) GSL. There was no significant effect on spectra of deuterons in the terminal Gal residue of gm_1.

The above findings were tested briefly and non-systematically after the incorporation of several additional factors that might be expected to influence the observations described: the divalent cation, Ca^{2+}, and protein. There is an extensive literature on metallic ion interactions with membranes (Rand & Parsegian, 1989; McLaughlin, 1989), and on ^2H NMR spectroscopy of phospholipid headgroup deuterium probes in the presence of ions (Akutsu & Seelig, 1981; Seelig & MacDonald, 1987; Seelig et al., 1987; Roux & Bloom, 1990). For instance, Akutsu & Seelig recorded that 10mM Ca^{2+} induced quadrupole splitting changes of up to 10-20% in wideline NMR spectra of deuterons in the choline headgroups of DPPC bilayers (1981). In recent years there has developed a specific interest surrounding the fact that divalent cations have been observed to encourage adhesion of carbohydrate-bearing liposomes - including those containing gangliosides or GalCer (Brewer & Thomas, 1984; Webb et al., 1988; Kojima & Hakomori, 1989; Hakomori et al., 1991; Stewart & Boggs, 1993b). The mechanism of this effect is unknown, and it represents too complex an area to consider in

detail in the present work; however the examples tested may provide insight into the design of future experiments. It was interesting that the spectral locations of the acetate peaks of gm_1 were found in the present work to be only modestly affected by Ca^{2+} concentrations that were up to 10 times physiological. Some broadening of spectral features was noted. However, since related spectral alterations were seen for asialo-gm_1, it is clear that the effect was not simply related to the presence of the negatively charged NANA residue.

In spite of early suggestions to the contrary, the affinity of Ca^{2+} ions for gangliosides is now known to be modest (McDaniel & McLaughlin, 1985). The overall Ca^{2+} binding constant is roughly the same for gangliosides as it is for anionic phospholipids, but the strong Ca^{2+}-mediated drive to lateral rearrangement often seen in the latter case has not been observed for gangliosides (reviewed in Raudino & Bianciardi, 1991). Raudino & Bianciardi (1991) have discussed this in terms of "diffuse" vs "compact" interfaces. Our observations seem to indicate that, for high concentrations of Ca^{2+}, there was some general alteration in GSL behaviour as minor components in fluid membranes, perhaps mediated through Ca^{2+} effects on the host matrix rather than through a specific effect on acidic glycolipids. The effect may be related to cation-induced alterations in hydration forces between the membranes studied (Rand & Parsegian, 1989). The situation is complicated

by the fact that neutralisation of the gm_1 net negative charge by Ca^{2+} could alter membrane spacing, as could binding of cations to an otherwise uncharged bilayer. Interestingly, the same conclusion - that Ca^{2+} had no measurable effect on preferred carbohydrate orientation - was reached in a recent detailed NMR study of a simple model glycolipid containing a NANA residue (Aubin & Prestegard, 1993). Goins et al. reported that 5 mM Ca^{2+} had no effect upon the lateral mobility of gm_1 labelled with a fluorescent probe on the NANA residue when dispersed in fluid DMPC (1986). Ca^{2+} effects on GSLs have been considered by Maggio et al. (1987), who concluded from DSC studies that variation in intermolecular packing was not involved, but that ion binding for both neutral and charged complex GSLs in DPPC was accompanied by modification in dipolar properties of the interface or in its state of hydration.

Membrane protein effects have been suggested by some workers as possible contributors to the phenomenon of GSL crypticity in cell membranes (reviewed in Hakomori, 1981). Such effects could be considered to arise from i) specific interactions, and ii) simple macromolecular 'crowding' (reviewed in Zimmerman & Minton, 1993). Unfortunately, the physical nature of lipid/protein systems, and their homogeneity, can be difficult to assess; so that it may prove extremely difficult to isolate contributory factors. Glycophorin was chosen in the

present work as an example of a membrane-spanning species with extensive carbohydrate (Marchesi et al., 1976). Bilayers containing glycophorin have been extensively characterised by our laboratory and other workers (Ketis & Grant, 1980; Ruppel et al., 1982; Grant & Peters 1984; MacDonald & Pink, 1987), and it is known to distribute uniformly as small oligomers in such systems. Glycophorin A is a 30 kd integral membrane protein that contains 16 negatively charged oligosaccharide chains (Vitala & Jarnefelt, 1985). Hence this species might be expected to alter GSL spectra by a number of mechanisms. It has been suggested that gm_1 may associate in bilayers with glycophorin (Umeda et al., 1984; Terzaghi et al., 1993). Serum albumin was selected as a common protein present at the outer plasma membrane surface (eg. Rechfeld et al., 1975) through its very high concentration in extracellular fluid. Spectral splittings were not seen to be altered in the presence of either protein. However, the glycophorin sample showed broader spectral lines. Whether this effect reflected some GSL/protein direct interaction, or an indirect effect mediated through bilayer changes will be very difficult to determine unambiguously.

5.5 CONCLUSIONS

The orientation and dynamics of simple and complex oligosaccharides of GSLs were examined using ^{2}H-NMR. It was found that

each of the oligosaccharides studied was observed to have a preferred average conformation and significant preservation of order. We are the first to find preferred carbohydrate orientation of a complex GSL as a minor component in a membrane environment. This was true in the face of various changes to the systems studied (see below). The independent wobble of the individual sugars on the oligosaccharide of the GSL, as well as the GSL as a whole, must be limited within the context of a fluid membrane.

The presence of the two species of gm_1 and asialo-gm_1, differing in sphingosine chain length, was not reflected in the spectra observed. Thus, it appeared that the 2-carbon difference in the sphingosine moiety of these two species had no effect on oligosaccharide conformation and dynamics. The naturally occurring variations in the fatty acids of GalCer and globoside had very modest effects on the spectra. These findings were similar to those of Chapter 4 which examined the effect of different fatty acids on the behaviour of GalCer. The removal of the negatively charged NANA residue, producing asialo-gm_1, had no significant effect on headgroup order or dynamics. No changes were noted in oligosaccharide orientation and order upon the introduction of a negatively charged species, sulphatide, to the membrane surface nor with the addition of approximately 23 mol% cholesterol. The effect of temperature on oligosaccharide behaviour seemed to be restricted to changes in order, while the host matrix was fluid.

Host matrix transition to a gel phase resulted in the gel phase features of the spectrum from the oligosaccharide deuterium labelled complex GSL.

In summary, it appeared that hydrophobic considerations, widely held to sensitively determine recognition site properties, do not seem to significantly influence oligosaccharide conformation and motion in a fluid regime. It would seem that alterations in headgroup conformation or extension from the membrane surface, as mechanisms regulating GSL crypticity, were not supported with the findings of this study within the parameters of the systems investigated here. The presence of protein, either transmembrane or surface, appeared to have no major effect on oligosaccharide properties. High concentrations of Ca^{++} resulted in the broadening of the spectral features perhaps reflecting non-specific ion interactions with the membrane surface. Due to the limited sensitivity of the experiments undertaken here, it cannot be ruled out that a very small fraction of the GSL population within a system might behave very differently to the majority species. The signal from a small population may be hidden by the dominant signal. It should also be noted that the wideline ^{2}H-NMR technique used in this study may not reflect phase-separated fluid domains or changes in lateral diffusion rates.

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