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A FOURIER-TRANSFORM INFRARED STUDY ON THE INTERACTION
BETWEEN SULFOGLYCOLIPID AND PHOSPHATIDYLCHOLINE.

by

Mayssa Attar

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in
partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa

Ottawa, Ontario, Canada

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ABSTRACT

The objective of this study was to investigate the interaction between the sulfoglycolipids, sulfogalactosylceramide (SGC) or sulfogalactosylglycerolipid (SGG), and dimyristoylphosphatidylcholine (DMPC) in a mixed model liposomal system (molar ratio 2:3). Structural and dynamic changes of the liposome contents were monitored by Fourier-transform infrared (FTIR) spectroscopy. Thermotropic FTIR analysis showed a single gel-to-liquid crystalline phase transition for SGC+DMPC liposomes (42°C) and SGG+DMPC liposomes (28°C). The thermotropic profile of SGG liposomes showed a pretransition followed by the main gel-to-liquid crystalline phase transition (45°C). Spectral changes of the amide and ester C=O bands arising from the functional groups at the interfacial region indicated a reduced hydrogen bonding of these groups in the mixed liposomes. Pressure-tuning FTIR of mixed SGC+DMPC liposomes showed that the methylene chains of SGC and DMPC were more orientationally disordered than those of the individual lipid liposomes. Analysis of SGG symmetric and antisymmetric CH₂ stretching bands in the gel phase and liquid crystalline phase revealed the insertion of DMPC into SGG bilayers results in an increase in the number of gauche conformers in the acyl chains as well a greater number of conformational states. Overall, these results suggested that the mixed liposomes of sulfoglycolipid and phosphatidylcholine consisted of a homogeneous mixture in which mutual shielding reduced hydrogen bonding at the interfacial region, with a concurrent increase in the orientational and conformational disorder of the hydrocarbon chains.

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LIST OF ABBREVIATIONS:

AS-A	Arylsulfatase-A
CaF₂	Calcium Fluoride
dDMPC	Deuterated Dimyristoylphosphatidylcholine
DMPC	Dimyristoylphosphatidylcholine
FAME	Fatty Acid Methyl Ester
FAB	Fast Atom Bombardment
FTIR	Fourier-transform Infrared
GC	Galactosylceramide
GG	Galactosylglycerolipid
GLC	Gas-Liquid Chromatography
HIV-1	Human Immunodeficiency Virus 1
HP-FTIR	High Pressure Fourier-transform Infrared
2D HMQC	Two Dimension Heteronuclear Multiple Quantum Coherence
IR	Infrared
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PAPS	3'-phosphoadenosine-5'-phosphate
PPG	Palmitoylglycerol
SGC	Sulfolactosylceramide
SGG	Sulfolactosylglycerolipid
T_m	Transition Temperature

TLC

Thin Layer Chromatography

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CHAPTER ONE

INTRODUCTION

1.1 Biomembranes

The plasma membrane is the cellular component that encloses the cytoplasm as well as controls the passage of molecules into and out of the cell. Therefore, the plasma membrane must be in a stable, although fluid state at physiological temperature (Singer and Nicolson, 1972; Shinitzky, 1984; Lipowsky, 1991; Bloom and Thewalt, 1994). Membranes of all cells, from bacteria to human, consist of phospholipids and glycolipids complexing with proteins where polar lipids form bilayer matrices into which the proteins are inserted (Singer and Nicolson, 1972). A great variety of lipids are found in mammalian membranes, including different hydrocarbon chains, polar groups, backbone structure (glycerol or sphingosine), types of chemical linkage (ester and/or ether) of the hydrocarbon chains to the lipid backbone, as well as other less common variations (Boggs, 1984, 1987). In prokaryotic and eukaryotic cells, glycolipids are located predominantly in the outer leaflet of the plasma membrane (Hakomori, 1984; Vos et al., 1994). Sulfated glycolipids have been implicated in numerous biomembrane functions, such as cation trapping and as modulators of cell adhesion and membrane fluidity (Murray and Narasimhan, 1990).

This thesis will examine the interaction of two sulfoglycolipids, in particular sulfogalactosylceramide (SGC) and sulfogalactosylglycerolipid (SGG) with a model membrane phospholipid. The aim of this study is to further elucidate the possible role(s) of these lipids in sperm membrane function.

1.2 Sperm and Fertilization

Mammalian sperm are synthesized via meiosis from spermatocytes, followed by special differentiation (the process termed spermiogenesis that is temperature sensitive) in the testis (Yanagimachi, 1994; Nolan and Hammerstedt, 1997). The whole process of sperm production in the testes is called spermatogenesis. Testicular sperm then migrate to the cauda epididymis, where they mature and gain the capability to move forwardly when released into culture media, but they do not have the capacity for fertilization. Sperm gain this ability through the capacitation process during their travel through the female reproductive tract (Yanagimachi, 1994). Capacitation involves a number of physiological and biochemical (functional) changes in the sperm that render them fertilization competent (Yanagimachi, 1994). The result of capacitation is that sperm gain binding ability to the zona pellucida (the egg extracellular matrix), acquire hyperactivated motility so they can penetrate through the zona pellucida, and undergo the acrosome reaction, which serves a dual function in rendering spermatozoa capable of (i) penetrating through the zona and (ii) fusing with the egg plasma membrane (Yanagimachi, 1994). The fertilizing capability gained during sperm capacitation is presumably a result of two important structural changes on the sperm plasma membrane. First, sperm plasma membrane lipid components are altered, including a decrease in the cholesterol:phospholipid ratio (Davis, 1981; Go and Wolf, 1983) and an elevated level of membrane-fusogenic lysophospholipids, resulting from an increase in phospholipase A2 activity (Langlais and Roberts, 1985). These lipid changes result in an observed increase in membrane fluidity (Wolf, 1986a). Second, membranous protein and sugar residues are modified, and this would presumably result in the removal of inhibitory

components and the exposure of egg receptors (Singer et al., 1985; Yanagimachi, 1994). There are also other changes during sperm capacitation, including: 1) higher adenylate cyclase activity (Hyne and Garbers, 1979; Breitbart and Spungin, 1997); and 2) a conversion of proacrosin to acrosin (Acrosin is a trypsin-like enzyme, the activity of which is believed to be pertinent to sperm penetration of the zona pellucida.) (Parrish and Polakoski, 1979; Brandon Jr. et al., 1997).

Capacitation leads to the acrosome reaction. The acrosome is a membrane bound, cap-like structure, derived from the Golgi apparatus of spermatocytes, located on the anterior portion of the sperm head. This organelle contains a large array of hydrolytic enzymes, including arylsulphatase-A (AS-A), hyaluronidase, β -N-acetylglucosaminidase and acrosin (Yanagimachi, 1994; Vos et al., 1994; Brandon Jr. et al., 1997). The acrosome reaction is a morphological change in the sperm head, which normally occurs at, or near, the zona pellucida and only acrosome reacted sperm are capable of passing through the zona pellucida and fusing with the egg plasma membrane (Yanagimachi, 1994; Brandon et al., 1997). Specifically, the acrosome reaction is a fusion event of the acrosomal outer membrane with the sperm plasma membrane that is triggered following sperm-zona pellucida binding. This results in membrane vesiculation and the release of the acrosomal contents containing hydrolytic enzymes which serve to digest the cumulus matrix and zona pellucida (Yanagimachi, 1994). Vasquez and Roldan (1997) have demonstrated that acrosomal exocytosis is associated with lipid changes in spermatozoa. This study revealed that phosphatidylcholine is hydrolyzed and resynthesized during the acrosome reaction and this metabolic pathway may be linked to changes in the level of diacylglycerol, a molecule known

to be involved in second messenger signalling.

1.3 Sperm Plasma Membrane

The sperm cell is a highly differentiated and polarized cell with topographically distinct domain structure (Nolan and Hammerstedt, 1997). The male germ cell plasma membrane must undergo a number of changes as it passes through different functional phases at different sites in the male and female reproductive tract. Initially, the testicular phase of spermatogenic cells requires a flexible membrane to allow for cell division and morphological specialization. Epididymal storage of sperm then requires a stable membrane in preparation for ejaculation. Finally, female tract passage of sperm requires selective but extended membrane destabilization in preparation for the "probability related event" of gamete interaction (Nolan and Hammerstedt, 1997).

The sperm plasma membrane has been found to have an unusually high amount of plasmalogens and other ether-linked lipids, as compared to somatic cells (Evans et al., 1980; Parks and Hammerstedt, 1985; Brouwers et al., 1998), as well as large proportions of long polyunsaturated aliphatic chains (Poulos, 1973; Evans et al., 1980; Nolan and Hammerstedt, 1997; Brouwers et al., 1998). Studies on sperm plasma membrane phospholipid content have revealed the most abundant species to be phosphatidylcholine plasmalogen, followed by phosphatidylethanolamine plasmalogen and sphingomyelin and only small amounts of phosphatidylserine and phosphatidylinositol (Evans et al., 1980; Parks et al., 1987; Parks and Lynch, 1992; Nolan and Hammerstedt, 1997; Brouwers et al., 1998). The phosphatidylcholine acyl chain composition of the sperm plasma membrane has been found to be unique compared to most mammalian cells. The predominant acyl chains associated

with the phosphatidylcholine plasmalogen class are C22:6, C22:5, C16:0, and C18:0, with the polyunsaturated species representing half or more of the total fatty acids present in the phosphatidylcholine fraction (Parks et al., 1987; Nolan and Hammerstedt, 1997; Brouwers et al., 1998). Brouwers et al. (1998) showed that the major phosphatidylcholine molecular species in boar sperm are 16:0/22:6 plasmalogen (33.2%) and 16:0/22:5 plasmalogen (22.4%). Nolan and Hammerstedt (1997) have suggested that the unique acyl and alkyl patterns of sperm membrane phospholipids may account for the unique fusogenic potential of sperm plasma membrane domains.

Biophysical studies have shown the existence of non-diffusing lipid domains in mature sperm membrane that may aid in sperm membrane regionalization, which is absent in the early part of spermatogenesis (Wolf et al., 1986a, 1986b, 1987). The presence of lateral phase segregation was demonstrated in the anterior region of the sperm head by differential scanning calorimetry which showed two endothermic transitions, an early transition at 26°C was suggested to be attributable to phospholipids and a later transition at 60°C was suggested to be attributable to glycolipids (Wolf et al., 1990).

1.4 Membrane Fluidity

Many important cellular, biochemical and biophysical events occur on the cell membrane and the membrane's components impart structural and dynamic features to the membrane that act as control mechanisms for these events (Shinitzky, 1984; Lipowsky, 1991). Therefore, the sperm plasma membrane's constituents may play a pivotal role in sperm function. In particular, the lipid hydrocarbon layer is the region sensitive to physical

and chemical effects on membranes, and the combination of positional, orientational, and motional freedom of the various hydrocarbon moieties will determine membrane fluidity (Shinitzky, 1984; Spector and Yorek, 1985). Normally, changes in the lipid composition of membranes are brought about either by translocation or exchange processes, or biosynthesis intracellularly and final processing at the membrane (e.g., oxidation of phospholipids or generation of lysophospholipids) (Shinitzky, 1984; Spector and Yorek, 1985). Sperm lipid components undergo dramatic changes during sperm maturation in the epididymis (Parks and Hammerstedt, 1985) and during capacitation in the female reproductive tract (Evans et al., 1980) (see section 1.2).

1.4.1 Modulators of Membrane Fluidity

Generally, some of the most important modulators of membrane fluidity include: 1) cholesterol; 2) double bond content; 3) sphingomyelin; and 4) phosphatidylethanolamine. Cholesterol under physiological conditions, above the lipid phase transition, acts as the main lipid rigidifier. However, below the lipid phase transition, in the gel phase, where normally a pure lipid bilayer is highly ordered, cholesterol acts in the opposite manner to increase fluidity and decrease order. Up to a certain level (i.e., cholesterol:phospholipid < 2), the cholesterol to phospholipid ratio can serve as a good qualitative parameter for correlation with the submacroscopic lipid microviscosity of the bilayer (Shinitzky, 1984; Mouritsen and Jørgensen, 1994). At abnormally high levels of cholesterol (i.e., cholesterol: phospholipid > 2) further addition of cholesterol to membranes has no effect, presumably due to lipid segregation (Shinitzky, 1984). The addition of a double bond acts to increase the space occupied by a lipid, thereby increasing disorder and membrane fluidity. On further addition

of double bonds this effect becomes progressively less pronounced (Shinitzky, 1984). Sphingomyelin and phosphatidylcholine comprise over 50 % of the phospholipid content in most animal membranes, and the phosphatidylcholine to sphingomyelin ratio influences membrane lipid fluidity (Shinitzky, 1984) by the virtue that phosphatidylcholine acts as a fluidizer and sphingomyelin as a rigidifier (Shinitzky, 1984; Huang and Mason, 1986). On the other hand, phosphatidylethanolamine's primary amine can form hydrogen bonds with adjacent phosphate groups or hydroxy groups, thus increasing the order of the membrane and decreasing fluidity (Shinitzky, 1984; Boggs, 1987).

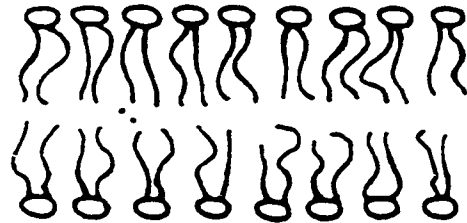
1.5 Lipid Organization in Lipid-Water Systems

Lipid molecules in an aqueous system will interact to form a variety of structures, which are classified as lamellar or non lamellar phases (Marsh, 1991; Tate et al., 1991). The different structures these lipids may adopt in water depend on the lipid species and composition, and the temperature (Marsh, 1991). The various phases can be classified according to three basic features: lattice type, chain order (fluid or extended chains) and in the case of curved structures, whether they are of the normal or inverted type (Marsh, 1991). Luzzati (1968) proposed the following nomenclature, an uppercase letter characterizes the lattice type, i.e., L, one-dimensional (lamellar); and H, two-dimensional (hexagonal). A lower case, Greek subscript is used to characterize the short range conformation of the hydrocarbon chains, i.e., α : disordered (fluid), or β : ordered and untilted (gel). When the interior and exterior can be distinguished topologically, Roman numerals are used to characterize the content of the structure element, i.e., Type I, paraffin in water (normal) or

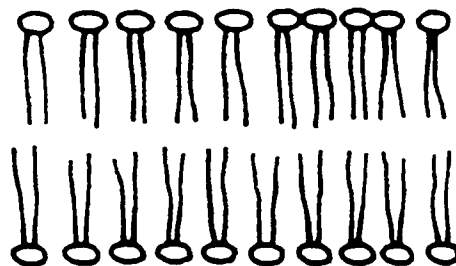
Type II, water in paraffin (inverted) (Marsh, 1991). One of the most biologically relevant phases is the lamellar phase, which consists of bilayer sheets of lipid stacked in a one dimensional lattice separated by layers of water (Shinitzky, 1984; Tate et al., 1991). The cross-sectional area per polar head group is close to that of the hydrocarbon chains of the lipid molecule. The lamellar phase can exist in different states or phases according to relative chain order/disorder. In the liquid crystalline phase, the hydrocarbon chains in the bilayer interior are highly disordered and are undergoing motional fluctuations as compared to the gel phase in which the bilayer exhibits a structure where the hydrocarbon chains are rigid and planar (Shinitzky, 1984; Marsh, 1991) (see Fig. 1.1). Another important structure is the hexagonal type II phase, where cylinders of water are embedded in lipid (see Fig. 1.1). The cross-sectional area per polar head group is smaller than the average cross section in the hydrocarbon chain (Shinitzky, 1984; Marsh, 1991). Metastable phases occur as lipids rearrange and re-order their hydrocarbon chains while changing from one stable lamellar phase to another. Various studies using differential scanning calorimetry and Fourier-transform infrared (FTIR) spectroscopy have examined the metastable behaviour of galactosylceramide (GC) (Curatolo et al., 1982, Curatolo, 1985; Jackson et al., 1988). These studies demonstrated that GC can exist in two different low temperature, gel phase polymorphs, one metastable and one stable. On heating, the metastable, GC arrangement undergoes an exothermic transition to the stable, low temperature, lower energy state and on subsequent heating undergoes an endothermic transition of this more stable gel state into the liquid crystalline (fluid acyl chain) state.

Figure 1.1 Illustration of lipid organizations in lipid-water systems. (A) Liquid crystalline phase; (B) Gel Phase; and C) Hexagonal type II phase. (Adapted from Shinitzky, 1984).

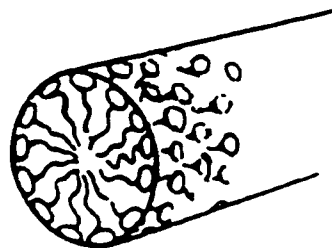
(A) Liquid Crystalline Phase



(B) Gel Phase



C) Hexagonal Type II Phase



1.6 Sulfogalactosylceramide

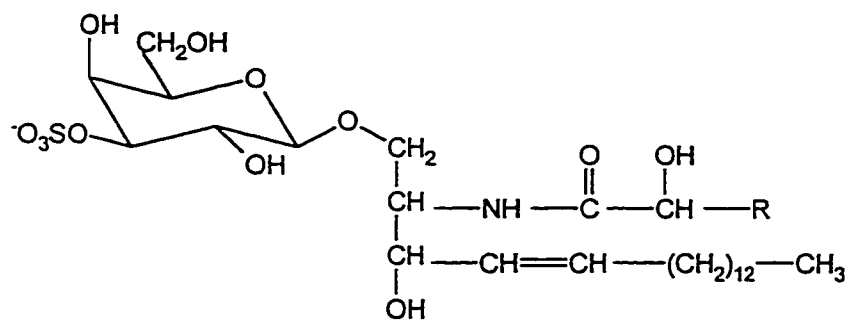
The major mammalian sulfatide, sulfogalactosylceramide (SGC), has a sphingosine backbone linked to monogalactosyl sulfate at C-1 position (Hakomori, 1983; Curatolo, 1987) (see Fig. 1.2).

SGC constitutes 4-7 mol % of total lipids in the myelin sheath (Murray et al., 1980; Murray and Narasimhan, 1990; Norton and Cammer, 1984) and is also found in the testes of birds, fish, reptiles, amphibians, and certain mammals including humans (Murray and Narasimhan, 1990) as well as in kidney and gastrointestinal epithelial cells (Hakomori, 1983).

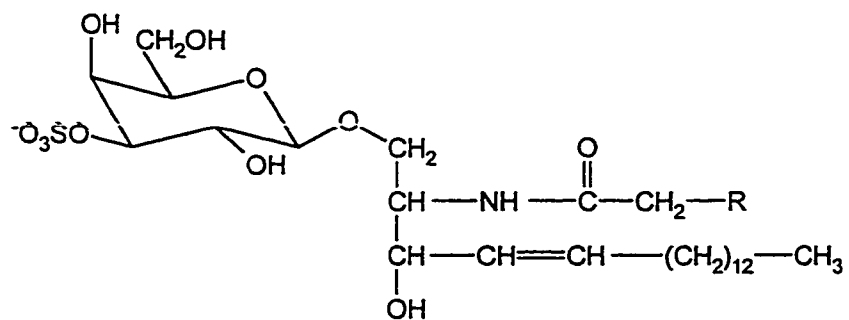
SGC was first discovered in 1884 by Thudichum, who isolated a sulfur containing lipid from the human brain and gave it the name sulfatide (Hakomori, 1983). SGC and its parent glycolipid GC exist naturally as acyl α -hydroxy and non-hydroxy species (Norton and Cammer, 1984). The structure of SGC was chemically characterized in 1933 by Blix who identified its constituents as sphingosine, fatty acid, galactose, and sulfate (Hakomori, 1983). The sulfate group was shown to be in ester linkage to the galactose 3-hydroxyl group (Yamakawa et al., 1962; Stoffyn and Stoffyn, 1963).

Figure 1.2 Chemical structures of SGC, SGG and DMPC. (A) α -hydroxylated SGC; (B) non-hydroxylated SGC; (C) SGG; and (D) DMPC.

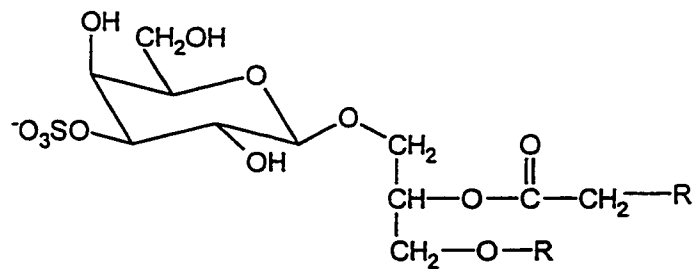
(A)



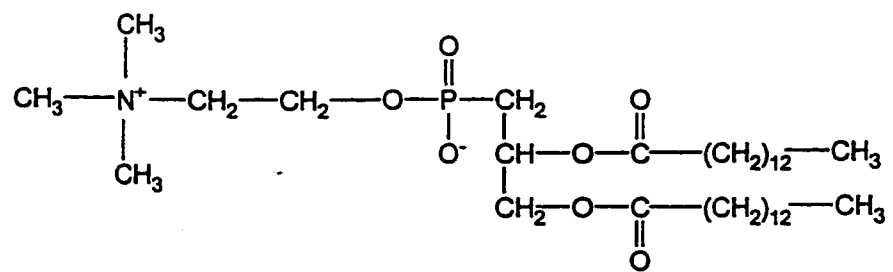
(B)



(C)



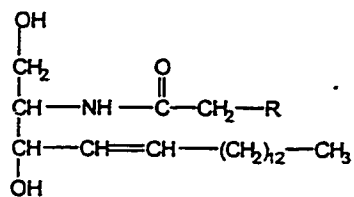
(D)



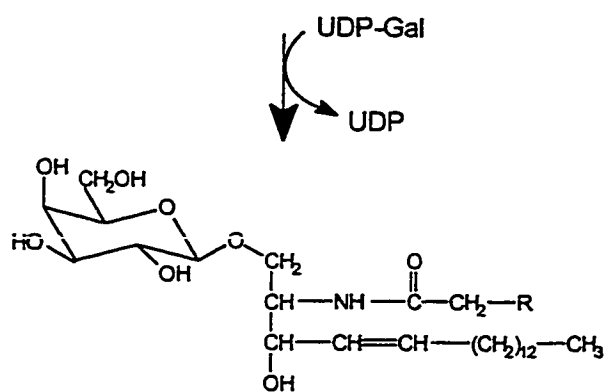
Synthesis of SGC is linked to the metabolic pathway of ceramides. Galactosyltransferase converts ceramide into GC to which sulfate is added by a sulfotransferase with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acting as the sulfate donor (Vos et al., 1994) (see Fig. 1.3). The final sulfation step of GC to generate SGC occurs in the Golgi apparatus, where a high level of sulfotransferase is also present (Fleischer and Zambrano, 1973).

The intramolecular and intermolecular hydrogen bonding of GC has been extensively studied by X-ray crystallography (Pascher, 1976; Pascher and Sundell, 1977). GC was shown to participate in lateral hydrogen bonding between the amide group and neighbouring sugar headgroups and the presence of the α -hydroxyl group on the fatty acyl chain results in increasing the degree of intramolecular and intermolecular hydrogen bonding (Pascher and Sundell, 1977). The increased degree of hydrogen bonding would result in a less fluid and hence more stable membrane and in accord with this, sphingolipid membranes exposed to physical and chemical stress are found to have a higher proportion of α -hydroxylated species (Pascher and Sundell, 1977). Furthermore, differential scanning calorimetry and infrared (IR) studies of SGC and GC bilayers indicate that α -hydroxylation of the acyl chain results in an increased transition temperature (T_m) resulting from an increased stability of the bilayers (Boggs et al., 1984, 1988, 1992).

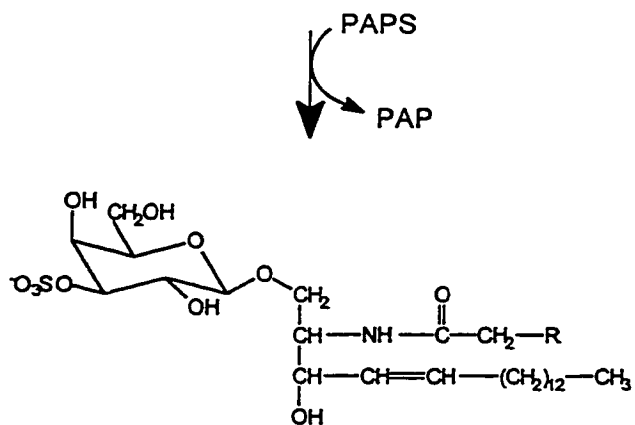
Figure 1.3 Schematic representation of SGC biosynthesis.



CERAMIDE



GALACTOSYLCERAMIDE



SULFOGALACTOSYLCERAMIDE

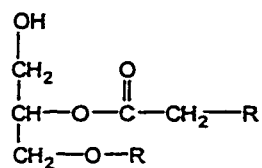
1.7 Sulfogalactosylglycerolipid

The presence of SGG in mammalian testis and spermatozoa was reported simultaneously by Kornblatt et al. (1972) and Ishizuka et al. (1973). Sulfogalactosylglycerolipid (SGG), commonly known as seminolipid, is the major glycolipid of mammalian male germ cells (5-8 mol % of total lipids). It is also found at a much lesser extent in the white matter of the brain (0.2 mol % of total lipids) (Murray and Narasimhan, 1990; Murray et al., 1980; Farooqui, 1981). Structure determination studies conducted by Ishizuka et al. (1973) showed that rat testis and boar testis and spermatozoa contain glyceryl ether, palmitic acid, galactose, and sulfate in approximately equimolar amounts. Various studies have since confirmed that SGG has the structure of a monoalkylmonoacylglycerol monogalactoside sulfate (see Fig. 1.2). Alvarez et al. (1990) using nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis for bovine testis SGG and Tupper et al. (1994) using gas-liquid chromatography (GLC) and IR spectroscopy analysis for ram testis SGG both confirmed Ishizuka's earlier structure determination results. The alkyl and acyl moieties were predominantly C16:0 (Ishizuka et al., 1973). The GLC analysis by Tupper et al. (1994) reported that ram testis SGG contains more than 95 % of the acyl group as C16:0, the remainder being C18:0, C14:0, and C14:1, and most of the alkyl groups consists of C16:0 (66 %), the remainder including C14:0 (7 %), C15:0 (2 %), C15:1 (7 %), C17:0 (1 %) and other minor components. IR, NMR and MS studies have established the β -anomeric configuration of the galactopyranoside, the presence of the sulfate at the 3'-position of the galactose moiety, and the attachment of the O-acyl group at the C-2 of the glycerol (Ishizuka et al., 1973; Alvarez et al., 1990; Tupper et al., 1994).

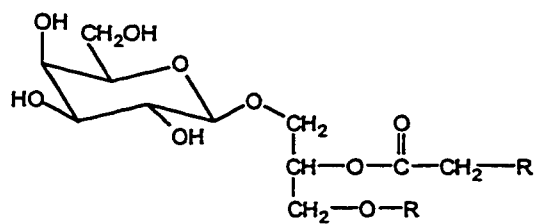
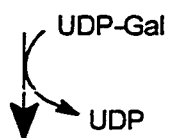
Studies by Kornblatt et al. (1974) have suggested that SGG is synthesized mainly in early spermatocytes. The synthesis of SGG occurs during the zygotene and early pachytene stages of spermatogenesis (Letts et al., 1978; Lingwood et al., 1985). Therefore, SGG is used as an early marker of male germ cell differentiation. A study conducted by Lingwood et al. (1994) suggested that germ cell SGG biosynthesis may be regulated via an insulin-like growth factor 1 mechanism acting on Sertoli cells. Using rat testis, Knapp et al. (1973) demonstrated enzymatic transfer of sulfate from PAPS to galactosylglycerolipid (GG) to form the sulfated SGG lipid (see Fig. 1.4). Furthermore, substrate competition between GC and GG suggested that the same PAPS-galactolipid sulfotransferase in rat testis acts on both galactosphingolipids and galactoglycerolipids. By analogy to other glycolipids, it is probable that GG biosynthesis occurs by galactosyl transfer from UDP-Gal to *sn*-1-alkyl-2-acylglycerol (Kates, 1990). SGG is synthesized in the Golgi bodies (Knapp et al., 1973) of zygotene spermatocytes (Lingwood et al., 1985) via GG sulfation (Knapp et al., 1973), and is then transferred to the outer leaflet of the germ cell plasma membrane, presumably via vesicular transport (Vos et al., 1994).

Once synthesized, SGG does not turn over and remains on the surface of live germ cells throughout their development to the spermatozoal stage (Kornblatt, 1979; Lingwood, 1985). Furthermore, SGG is stable during capacitation (Tanphaichitr et al., 1990) and turns over only when sperm become senescent (Kornblatt, 1979; Tanphaichitr et al., 1990).

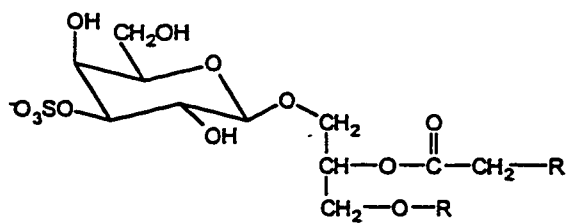
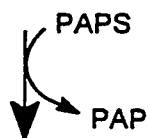
Figure 1.4 Schematic representation of SGG biosynthesis.



1-ALKYL-2-ACYL-GLYCEROL



GALACTOSYLGlycerolIPID



SULFOGALACTOSYLGlycerolIPID

Isolated SGG and SGC have been shown to serve as natural substrates of AS-A (EC 3.1.6.8, sulfate hydrolyzing enzyme) and this enzyme has been purified from boar testes by Yamato et al. (1974). Human and mouse testis AS-A have been cloned (Stein et al., 1989; Kreysing, 1994). AS-A appears to be localized on the sperm head surface in the same region as SGG (Nikolajczyk and O'Rand, 1992; White et al., 1998), although there is no apparent sign of SGG conversion to GG throughout spermatogenesis, sperm maturation and the initial stage of sperm capacitation (Kornblatt, 1979; Lingwood, 1985; Tanphaichitr et al., 1990). This is presumably due to the absence of saposin B, an AS-A co-activator protein, which configures the sulfoglycolipid in the proper conformation to the enzyme (Fischer et al., 1978). Presumably, this small activator protein, saposin B, is complexed to SGC and "solubilizes" the hydrophobic substrate (Lukatela et al., 1998). Lukatela et al. (1998) have recently determined the crystal structure of human AS-A, which reveals a high degree of similarity to alkaline phosphatase from *E. coli*. The active site was described as containing amino acid cysteine 69 oxidized to formylglycine and being lined mainly with charged amino acids from both the C-terminal and N-terminal end, i.e., lysine, histidine and serine (Lukatela et al., 1998). In contrast, a hydrophobic domain near the active site is not present, and this suggests and supports the role of saposin B as a co-activator enzyme to AS-A through interaction with the hydrophobic chains of SGC. During the latter phase of in vitro capacitation 15% of SGG is desulfated (Tanphaichitr et al., 1990). While saposin B has not yet been shown to be present with SGG in vivo, prosaposin has been documented to be present in the male reproductive tract (Hermo et al., 1992) including the caudal epididymal fluid (White et al., unpublished results) and in quantity in the seminal plasma (Hiraiwa et al.,

1993). Prosaposin could be absorbed onto the sperm surface. Unpublished indirect immunofluorescence results from our laboratory in fact reveal that prosaposin is present on the sperm surface of mouse caudal epididymal sperm, mouse uterine sperm and ejaculated human and pig sperm.

Our laboratory has proposed a mechanism for SGG metabolism following sperm-zona pellucida binding. Since SGG is present in significant amounts on the anterior head of sperm (up to 25 %, B. Gadella, personal communication), it would be expected that its bulky polar head group, galactosyl sulfate, would hinder membrane fusion, a requirement for the acrosome reaction. We have, therefore postulated that SGG may be desulfated by AS-A and saposin B to yield GG, which may then may be further degalactosylated by β -galactosidase, yielding palmitoylglycerol (PPG). Being a diradylglycerol, PPG may activate sperm phospholipase A₂ (Roldan and Fragio, 1994) to hydrolyze phospholipids, yielding fusogenic lysophospholipids and free fatty acids. These end products would enhance membrane fusion essential for initiation of the acrosome reaction. This postulation is based on the fact that: 1) hydrolysis of SGG's sulfate group (~ 15 % of total SGG) occurs at the same time as a spontaneous acrosome reaction (~ 15 % of total sperm population) (i.e., after mouse sperm are capacitated for a prolonged period of time, Tanphaichitr et al., 1990); and 2) both AS-A and β -galactosidase are present on the sperm surface (Nikolajczyk and O'Rand, 1992; Tulsiani et al., 1995). This postulated metabolic pathway of SGG is currently being investigated in our laboratory. It should also be mentioned that GG itself, like other monogalactolipids, can induce the transition of the lipid bilayer from the lamellar to the hexagonal II phase, which is proposed as an intermediate in membrane fusion (Curatolo,

1987). Thus, GG formed from SGG in the sperm head may also be directly involved in the acrosome reaction and gamete membrane fusion (Gadella et al., 1991, 1992).

1.8 SGC and SGG as Structural Analogs

SGC and SGG possess a number of common structural features (see Fig. 1.2), such as: 1) a negatively charged galactosyl-3-sulfate group, which allows interaction with cations and basic amino acids of proteins in the extracellular milieu; 2) an ester carbonyl C=O group in SGG and an amide C=O group in SGC, which can participate in hydrogen bonding; and 3) long hydrocarbon chains, as part of the sphingosine backbone and amide fatty acyl group in SGC or in ether linkage and ester linkage at the *sn*-1 and *sn*-2 glycerol positions, respectively, of SGG (Vos et al., 1994). Therefore, it is not surprising that all antibodies produced against SGC also cross-react with SGG and vice versa (Lingwood et al., 1980; Crook et al., 1987; Fredman et al., 1988; Harouse et al., 1991; Gadella et al., 1994; White et al., 1998).

1.9 Functions of SGC and SGG

SGC, which is commercially available, is a more extensively studied sulfoglycolipid than SGG. Most of the postulated biological functions of SGG are based on speculation by analogy to known functions of SGC.

1.9.1 Cation Trapping

The negatively charged sulfate group in SGC and SGG may play a role in cation trapping and in subsequent cation transport into cells. Studies demonstrating direct binding

of SGC (Abramsom et al., 1967; Tupper et al., 1992) or SGG (Tupper et al., 1994) to cations have been reported. Kidney epithelial cells, which are involved in ion transport, contain more SGC when cultured in high ionic strength medium than when kept in normal medium (Karlsson, 1974; Ishizuka and Nakamura, 1991). SGC also influences (Na⁺/K⁺)ATPase activity by modulating K⁺ binding (Jedlicki and Zambrano, 1985; Rintoul and Welte, 1989). In addition, the parent glycolipid, GC, also participates in the opening of voltage-insensitive Ca²⁺ channels of oligodendrocytes (Dyer and Benjamins, 1990).

1.9.2 Cell Adhesion

SGC and SGG, being located on the outer leaflet of the plasma membrane, are also engaged in cell-cell recognition, cell-substratum recognition, and ligand-receptor interaction (Curatolo, 1987). SGC and SGG, as well as their respective parent glycolipids, GC and GG, have been shown to interact in vitro with gp 120, the surface glycoprotein of HIV-1 (Harouse et al., 1991). This may be the basis of one of the molecular mechanisms of neuronal virus transfection, i.e., via viral binding to the glycolipids present in the myelin sheath surrounding the neuronal axons. Human and animal influenza viruses bind SGC, and the exogenous addition of this sulfoglycolipid inhibits infection of Madin-Darby canine kidney epithelial cells by human influenza virus 1 (Suzuki et al., 1996). Also, the binding of infertility related mycoplasmas to SGC and SGG in vitro (Lingwood et al., 1990) suggests that SGG and SGC present in human sperm may be the receptors of these mycoplasmas in vivo. In our laboratory, we have recently shown that mouse sperm (which contain only SGG as their sulfoglycolipid in the plasma membrane overlying the sperm head and midpiece) pretreated with affinity purified antiSGG IgG antibody or partially purified AS-A (presumably

containing saposin B) lose their ability to bind to the zona pellucida (Kamolvarin et al., 1996; White et al., 1998). Laminin, an egg extracellular matrix protein, also binds to SGC or SGG in vitro (Roberts et al., 1985). This observation supports the concept that extracellular matrix proteins (or extracellular ligands) interact with a cell surface receptor that is lipid, or protein-lipid. Consistent with this finding, SGC and SGG have been shown to be associated with opiate receptors (Loh et al., 1978).

1.9.3 Modulators of Membrane Fluidity

Ishizuka and Tadano (1982) observed that Ca^{2+} has a high affinity for SGC and presumably this also applies to SGG (Murray and Narasimhan, 1990). High-pressure Fourier transform infrared (HP-FTIR) studies in our laboratory on SGG and SGC liposomes revealed that Ca^{2+} binds to the sulfate group of these sulfoglycolipids, resulting in disruption of the interdigitated state of the SGG and SGC hydrocarbon chains and thus an increase in SGG and SGC bilayer fluidity (Tupper et al., 1992, 1994).

The presence of ether bonds in ethanolamine glycerophospholipids (e.g. plasmalogens) has been shown to slightly increase the gel-to-liquid crystalline phase T_m and significantly decrease the liquid crystalline-to-hexagonal type II phase transition temperature (Paltauf, 1994). This may be through destabilization of the lamellar phase as reported by Boggs et al. (1981) for alk-1'-enyl or alkyl chains in position *sn*-1 of phosphatidylethanolamine lipids. SGG contains an ether bond which is more stable than an ester bond and may perhaps have an effect on membrane dynamics similar to that of phosphatidylethanolamine species having ether bonds.

1.10 Fourier-Transform Infrared Spectroscopy

1.10.1 Theory of Infrared Spectroscopy

Virtually all compounds possessing covalent bonds are found to absorb various frequencies of electromagnetic radiation in the infrared spectral region (Pavia et al., 1979). The energy of a molecule consists partly of translational energy, partly of rotational energy, partly of vibrational energy, and partly of electronic energy (Colthup et al., 1990). Electromagnetic radiation is characterized by its wavelength (the length of one wave), its frequency (the number of vibrations per unit time), and its wavenumber (the number of waves per unit length). The vibrational portion of the infrared region is defined as including radiations with wavelengths between 2.5 μ and 15 μ (Pavia et al., 1979). It is the vibrational energy that is specific to a compound's particular molecular vibration that gives rise to its characteristic absorption bands in the infrared region of the electromagnetic spectrum (Pavia et al., 1979; Colthup et al., 1990). Vibrational spectra of lipid systems consist of bands arising from the transitions between vibrational energy levels of various types of intramolecular vibrations in the ground electronic state (Reis et al., 1996). The absorption of infrared radiation is a "quantized" process such that only selected frequencies (energies) of infrared radiation will be absorbed by a molecule (Pavia et al., 1979). Those frequencies of infrared radiation, which match the natural vibrational frequencies of the molecule being examined may be absorbed, and the energy absorbed will serve to increase the amplitude of the vibrational motions of the bonds of the molecule (Pavia et al., 1979). Every type of bond has a different natural frequency of vibration, and since the same type of bond in two different compounds is in a slightly different environment, no two molecules of different

structures will have the same infrared absorption pattern. Thus, IR spectroscopy can be used to characterize a molecule at a structural group level. Spectral parameters such as frequency, width, intensity, shape and splitting of infrared bands are sensitive to the structural and dynamic properties of membrane lipid molecules (Wong et al., 1988).

Stretching and bending modes are the simplest types of molecular vibrational modes, which are infrared active (Pavia et al., 1979). Stretching vibrations consist of periodic stretching of an A-B bond along its axis, while bending vibrations of an A-B bond are displacements that occur at right angles to the bond axis (Pavia et al., 1979).

Fourier-transform infrared (FTIR) spectroscopy is essentially the only method of IR study now employed since non FTIR (dispersive) spectrophotometers are no longer manufactured (Larrabee and Choi, 1993). The most important advantage that FTIR spectrophotometers have over dispersive spectrophotometers is the large signal-to-noise ratio. In a dispersive instrument the intensity of each resolution element of wavelength or wavenumber is measured one by one in sequence and only a small spectral range falls on the detector at any one time. In an FTIR instrument, the intensities of all the resolution elements are measured simultaneously by the detector. Fourier transform analysis of an infrared spectrum involves computerized mathematical manipulation (i.e., Fourier transform) of the spectroscopic data collected to give more accurate frequency determination. Furthermore, visualization of overlapping bands is possible through band narrowing mathematical manipulations, such as Fourier self deconvolution and derivation, that result in band separation of underlying bands (Mantsch and McElhaney, 1991). Other important advantages of an FTIR spectrophotometer include speed, more efficient use of radiation and

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built in data processing abilities (Harris, 1987).

FTIR spectroscopy has proven to reveal structural information on lipids and proteins. However, due to the large size of proteins, the information collected by FTIR spectroscopy is of low structural detail. In contrast, smaller lipid molecules do not pose this problem (Surewicz et al., 1992). FTIR spectroscopy has a number of advantages for the study of lipid systems in particular (Mantsch and McElhaney, 1991) including: 1) FTIR spectroscopy provides structural and dynamics information from all regions of the lipid molecule simultaneously in a single experiment (Mantsch and McElhaney, 1991); 2) Molecules are not required to be labeled or modified in FTIR spectroscopy (Lewis and McElhaney, 1990); and 3) Studies of intact membranes and living cells as well as model membranes are possible (Auger et al., 1987; Tupper et al., 1992, 1994; Wong, 1993).

1.10.2 Use of CH₂ Stretching for Thermotropic Studies

Thermotropic FTIR can be used to monitor the physical state of lipid bilayers. The CH₂ stretching vibrations, involving a shift towards higher frequencies and broadening of the 2920 cm⁻¹ (antisymmetric stretching) and 2850 cm⁻¹ (symmetric stretching) bands with increasing temperature, are employed to determine the T_m (Nabet et al., 1996). These carbon-hydrogen stretching vibrations are particularly useful for monitoring the melting of the hydrocarbon chains because the CH₂ antisymmetric and symmetric stretching modes are sensitive to temperature induced changes in conformation of the hydrocarbon chains. The CH₂ symmetric stretching band is usually monitored because it is relatively free from overlap with other vibrational modes, such as those from antisymmetric and symmetric stretching of the terminal CH₃ group (Mantsch and McElhaney, 1991).

1.10.3 Use of C=O Stretching for Assessing Interfacial Hydrogen Bonding

The carbon-oxygen vibrations are useful for probing the sphingosine or glycerol-acyl chain interface (Mantsch and McElhaney, 1991). The degree of interfacial hydrogen bonding can be assessed by examining the amide I stretching mode (ranging from 1630-1670 cm^{-1}) and the ester C=O stretching mode (ranging from 1700-1750 cm^{-1}) (Kates, 1986). Wong and Mantsch (1988) have shown previously that the frequencies of these two modes decrease proportionally with the degree of their hydrogen bonding to proton donating groups, due to the elongation of the C=O bonds. Therefore, a shift in these stretching frequencies in mixed lipid liposomes, as compared to that in individual lipid liposomes, can be used to monitor changes in interfacial hydrogen bonding.

1.10.4 Use of CH₂ Bending for Barotropic Studies

Pressure-tuning FTIR spectroscopy is used to evaluate the dynamic properties of lipid bilayers. It has been demonstrated that the conformational disorder, the reorientational fluctuations, and the torsion/twisting motions of methylene chains in lipid bilayers can be ordered and dampened by external pressure (Wong, 1994). Application of pressure to lipid bilayers reduces the mobility of the hydrocarbon chains resulting in a higher degree of order (Wong, 1984). As the orientation of each hydrocarbon chain becomes more ordered and provided that the plane of each hydrocarbon chain becomes nearly perpendicular to its neighbouring chain (i.e., nonequivalent), this phenomenon spectroscopically results in correlation field splitting in the vibrational modes of the hydrocarbon chains (Auger et al., 1988, 1990; Tupper et al., 1994). The correlation field splitting of these modes originates from the vibrational-coupling-interchain interactions between fully extended methylene

chains with different site symmetry along each bilayer leaflet (Wong, 1994). The pressure at which this correlation field splitting is initially observed is directly related to membrane fluidity (Wong et al., 1988; Wong, 1994). Furthermore, by increasing pressure on a sample, interactions which are normally weak at ambient pressure increase in magnitude and may eventually be monitored (Wong, 1994). Interchain distances and the relative orientation of the methylene chains in lipid bilayers are varied with pressure and the resultant frequency shifts and correlation field splitting are monitored to study structural changes in lipid bilayers. The pressure reorients the hydrocarbon chains and reduces the number of gauche conformers along the acyl chains, which results in increased order in the structure of the chains and thus in increased intermolecular interaction potential (Reis et al., 1996). The methylene bending mode absorption band (CH_2) (1450 to 1490 cm^{-1}) is usually used for this purpose, since it is sensitive to structural differences in hydrocarbon chain packing (Stein and Sutherland, 1953, 1954).

Experimentally, the sample is placed in a diamond anvil cell, which can withstand high pressures, during the HP-FTIR experiment, and the hydrostatic pressure within the gasket of the cell is measured with computerized software by monitoring the pressure shift of the infrared bands of the internal pressure calibrant, i.e., quartz (Wong et al., 1985).

1.10.5 Use of CH_2 Stretching to Assess Hydrocarbon Chain Order

The dynamics and molecular order of the hydrocarbon chains can also be assessed using the symmetric and antisymmetric methylene CH_2 stretching bands. These C-H stretching vibrations are characteristic of the hydrophobic hydrocarbon region (Mantsch and McElhaney, 1991). In particular, the symmetric CH_2 stretching band, which is conformation

sensitive, is related to the average number of gauche conformers in a system; thus, when hydrocarbon chains are in an all-trans conformation these methylene groups absorb at $\sim 2849\text{ cm}^{-1}$ and the introduction of gauche conformers results in an increase in this absorption frequency (Umemura et al., 1980; Mantsch and McElhaney, 1991; Reis et al., 1996). Furthermore, changes in bandwidths can be related to variations in the motional rates of the acyl chains (Cameron et al., 1980; Umemura et al., 1980; Mantsch and McElhaney, 1991).

1.11 Research Objectives

The potential role of sulfoglycolipids in modulating biomembrane dynamics and function is supported by their location on the outer leaflet of the plasma membrane, negative charge (which can trap cations) and selective abundance in membranes of certain tissues/cells. Pressure-tuning FTIR spectroscopy performed in our laboratory indicated that divalent cations, such as Ca^{2+} , bind to SGC and SGG model membranes with a higher affinity than monovalent cations, such as Na^+ , and this divalent cation binding results in increased membrane fluidity of the SGC or SGG liposomes (Tupper et al., 1992, 1994). However, the question still remained as to whether SGC or SGG, itself, can influence fluidity of other co-existing lipids within the membrane or can be influenced by other co-existing lipids within the membrane. The present research project was designed as a first approach to determine this possibility. Therefore, we examined by FTIR spectroscopy a mixed model liposome system consisting of SGC or SGG and dimyristoylphosphatidylcholine (DMPC, a model membrane phospholipid) (see Fig. 1.2) in a 2:3 molar ratio. This ratio is only a first attempt at approaching a physiologically relevant ratio. It was chosen since our initial model

studies conducted using SGC at a lower ratio failed to reveal the C=O band of the amide bond. Commercial SGC contains several molecular species. Therefore, in order to obtain a strong IR signal for the amide C=O band of SGC in mixed liposomes with DMPC, the 2:3 ratio was found to be experimentally suitable. DMPC is an extensively studied phospholipid and was selected as a model phospholipid. Furthermore, it is available commercially in fully deuterated form, thus allowing selective examination of the order/disorder of SGC or SGG hydrocarbon chains in the mixed liposomes. In this study, we determined whether interaction between the hydrocarbon chains of SGC or SGG and DMPC resulted in changes of hydrogen bonding at the interfacial region of the liposomes and/or increased disorder (fluidity) of the hydrocarbon chains of the liposomes.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Bovine brain SGC (containing both α -hydroxy and non-hydroxy fatty acids; major cations were 1.72% Na^+ , 1.07% Ca^{2+}), DMPC, Bio-Sil A silica (bead size 150-300 μm) and tris-(hydroxymethyl)aminomethane (Tris) hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). DMPC (fully deuterated in both hydrocarbon chains) and standard fatty acids and fatty acid methyl esters (FAMES) for GLC were purchased from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Silica gel thin-layer chromatography (TLC) plates (K6 silica gel 60 Å, 250 μm layer, 20 x 20 cm) were obtained from Whatman (Kent, England). D_2O (99.9% atom %) was purchased from Merck Sharp and Dohme/Isotopes (Pointe-Claire, PQ, Canada). High-purity natural crystalline α -quartz was obtained from Karl Lambrecht Corp. (Chicago, IL). Cerotic (26:0) acid was purchased from Eastman Kodak (Rochester, NY) and α -hydroxy cerotic acid (C26:0-h), isolated from yeast cerebrin, was a gift from Dr. N. Z. Stanacev (University of Toronto, Toronto, ON, Canada). Synthetic non-hydroxy C24:1 SGC was a gift from Dr. J. M. Boggs (Hospital for Sick Children, Toronto, ON, Canada).

2.2 Analysis of Total Fatty Acids of SGC

The non-hydroxy fatty acid moieties of SGC were analyzed as FAMES and the α -hydroxy fatty acids were analyzed as methylated or acetylated FAMES on a GLC column (2

m, length; 4 mm, diameter) of 10% SP2330 on Gas-Chrom A (Supelco, Bellefonte, PA) at 230°C with a N₂ inlet pressure of 1.2 kg/cm², using a PYE Unicam instrument, as previously described (Kates, 1986). SGC was subjected to methanolysis in 2 N methanolic HCl in a screw cap tube at 80-90°C for 5 to 6 h. The FAMES were extracted with petroleum ether and a portion was analyzed by GLC (Kates, 1986). A second portion was subjected to methoxylation in iodomethane/toluene (1:1, v/v) in the presence of silver oxide and calcium sulfate at 50 to 60°C overnight in screw cap tubes. After dilution of the reaction mixture with ethyl ether and removal of silver and calcium salts by centrifugation, the ether solution was brought to dryness under a stream of N₂ and the residual methoxy-FAMES were dissolved in CHCl₃. A third portion of the FAMES was subjected to acetylation in acetic anhydride and pyridine (anhydrous) (1:2, v/v) at 40°C for 1 h (Kates, 1986). Acetoxy-FAMES were extracted with petroleum ether, brought to dryness under a stream of N₂ and dissolved in CHCl₃. Methoxy-FAMES and acetoxy-FAMES were analyzed by GLC as described for FAMES.

2.3 Extraction and Purification of SGG

Extraction of lipids from ram testes was carried out using the Bligh and Dyer method (1959) as modified by Kates (1986). A pair of ram testes (~ 350 g each) were obtained from the C. H. Thomas Slaughterhouse (Ottawa, ON). Tunica albuginea, the enveloping connective tissue of each testis, was removed and the testis was cut into small pieces. One hundred gram portions of testes tissue was homogenized in 150 ml MeOH:CHCl₃ (2:1 v/v) in a Waring blender for 3 min at top speed at room temperature. The homogenate was then

suction-filtered on glass wool using a Buchner funnel. The ratio of MeOH:CHCl₃:H₂O in this single phase filtrate was close to 2:1:0.8 (v/v/v) assuming 85% water content in the tissue. Equal volumes of CHCl₃ and H₂O were added to the single phase filtrate to give a two phased system with a final ratio of MeOH:CHCl₃:H₂O (1:1:0.9, v/v/v). The two phased system was gently mixed, then transferred into a separatory funnel and left overnight at room temperature to allow complete separation of the two phases. The lower CHCl₃ phase containing the lipids was transferred to a round bottom flask, diluted with benzene and concentrated almost to dryness using a rotary evaporator. The concentrated CHCl₃ phase was transferred to a centrifuge tube and diluted with ten volumes of acetone and left overnight at -10°C. The precipitate of glycolipids and phospholipids was centrifuged briefly at low speed. The supernatant (containing neutral lipids) was removed with a Pasteur pipette and the pellet was washed once with 0.5 ml acetone and dried under an N₂ stream and brought up in CHCl₃. The extracted lipids in CHCl₃ were fractionated by adsorption column chromatography using Bio-Sil A silica (Kates, 1986). The column consisted of 60 g of silica with a column volume (80 ml) onto which 800 mg of lipid was loaded. The lipids were eluted using the following organic solvents respectively: CHCl₃, 1 column volume; CHCl₃/acetone (1:1, v/v), 1 column volume; acetone, 15 column volumes; and MeOH, 10 column volumes. Lipid content of each fraction was monitored by TLC using the solvent system CHCl₃:MeOH:H₂O, 65:25:4 (v/v/v) with authentic phospholipid and glycolipid standards. The thin-layer chromatogram was visualized with iodine vapor and α -naphthol/H₂SO₄ staining, as described in section 2.4.2. The acetone eluates containing mainly SGG were then combined, brought down to dryness in a rotary evaporator, and the

SGG residue was dissolved in a small volume of CHCl_3 . SGG was then precipitated with addition of acetone to the final acetone: CHCl_3 ratio of 10:1. The SGG precipitate was centrifuged down and purified by preparative TLC (see section 2.4.1).

2.4 Thin Layer Chromatography

Thin layer chromatography (TLC) plates were developed in a neutral solvent system, CHCl_3 :MeOH: H_2O (65:25:4, v/v/v) that effectively separates glycolipids from other lipids (Ishizuka et al., 1973), using authentic phospholipid and glycolipid standards. TLC plates were washed twice in CHCl_3 :MeOH (1:1, v/v) and activated by heating in an oven at over 100°C for one hour.

2.4.1 Preparative TLC of SGG

Preparative TLC was used as a final step in the isolation and purification of SGG. The lipids collected in the acetone fraction from the silica adsorption column (see section 2.3) were brought down to dryness in a 100 ml round bottom flask on a rotary evaporator. The lipid residue was brought into solution with CHCl_3 and transferred to a 15 ml stoppered centrifuge tube with a Pasteur pipette. This lipid solution was brought down to a small volume (~ 0.2 ml) with a N_2 stream. The solution was then spotted onto an activated TLC plate in a long band (~ 15 cm) along the bottom of the plate. SGG standard was spotted on either side of the sample band. Once the plate had developed, it was left to air dry for 5 min and then stained with iodine. The band corresponding to the standard SGG was marked on the silica plate and then scraped off onto weighing paper. The scraped silica was then transferred to a 50 ml centrifuge tube and 6 ml neutral Bligh and Dyer solvent

(MeOH:CHCl₃:H₂O, 2:1:0.8, v/v/v) was added. The solution was vortexed vigorously and left to extract overnight at room temperature. The next day the solution was centrifuged briefly at low speed and the supernatant was transferred to a 50 ml centrifuge tube with a Pasteur pipette. The silica pellet was washed twice with neutral Bligh and Dyer solvent. The pooled neutral Bligh and Dyer extracts were subjected to an acid Bligh and Dyer partition (see section 2.5) and the resulting SGG free acid was neutralized with 1 N NH₄OH.

2.4.2 Visualizing lipids on TLC plates

TLC plates were first stained with iodine vapor. The developed TLC plate was placed in a closed container saturated with iodine vapor for a few minutes (Kates, 1986). All lipids were detected as yellow-brown spots. Glycolipids on the analytical TLC plates were detected by α -naphthol staining. Briefly, the TLC plate was sprayed with a 0.5% α -naphthol solution (0.5 g of α -naphthol in 100 ml of methanol-water (1:1, v/v)) and then allowed to air dry for a few minutes. The TLC plate was then sprayed lightly with concentrated sulfuric acid (sulfuric acid-water (95:5, v/v)) and heated in an oven at 120°C until glycolipids appeared as blue-purple spots (Kates, 1986). Upon further heating neutral lipids and phospholipids appeared as yellow-brown spots while cholesterol appeared as a red spot. The presence of a sulfate group was confirmed with Azure A /H₂SO₄ staining of analytical TLC plates. The plate was sprayed with Azure A (92%)/H₂SO₄ solution (40 mg Azure A dissolved in 250 ml 0.05 N H₂SO₄). The plate was then washed three times with fresh 0.04 M H₂SO₄/ MeOH (3/1, v/v). The plate was then air dried and visualized under UV light, sulfated glycolipids appeared as blue-white spots.

2.5 Replacement of Endogenous Cations from DMPC, SGC, SGG, SGC+DMPC and SGG+DMPC with Sodium Ions

The acid Bligh and Dyer procedure (Bligh and Dyer, 1959) as modified by Kates (1986) was used to remove any cations from DMPC, SGC, SGG, SGC+DMPC and SGG+DMPC. To a solution of 2 mg lipid in 2 ml of MeOH:CHCl₃ (1:1, v/v), aqueous 0.2 N HCl was added to give a MeOH:CHCl₃:HCl ratio of 1:1:0.9 (v/v/v). The mixture was inverted gently and briefly centrifuged at a low speed. The upper MeOH-H₂O phase was removed and the lower CHCl₃ phase was washed twice with fresh upper phase (MeOH:H₂O, 10:9, v/v) to remove any traces of HCl. The CHCl₃ phase was titrated to neutrality with methanolic 0.05 N NaOH and then brought down to a small volume (~0.3-0.5 ml) under a stream of N₂. This solution was diluted with a tenfold amount of acetone and left overnight at -10°C. The precipitate of the sodium salt of the sulfoglycolipids was centrifuged briefly at a low speed. The supernatant was then removed with a Pasteur pipette and the pellet was washed once with 0.5 ml acetone and vacuum dried in a desiccator over KOH pellets at 10 mm Hg pressure.

2.6 NMR Analysis

The Bligh and Dyer (1959) method as modified by Kates (1986) was used to prepare the SGG sample in ammonium salt form for NMR analysis. The dried lipid was dissolved in NMR solvent (CDCl₃) and filtered through Kimwipes into an NMR microtube.

Spectra were obtained with a Bruker AMX 500 spectrometer using a standard one pulse experiment. The two dimensional (2D) spectra were 2D heteronuclear multiple

quantum coherence (2D HMQC) run with a standard Bruker pulse program. Chemical shifts are expressed in ppm and referenced to tetramethylsilane at 0 ppm.

2.7 Mass Spectrometry Analysis

Negative fast atom bombardment mass spectrometry (FAB)-MS analyses on intact SGG were carried out using a Kratos Concept IIH mass spectrometer fitted with a cesium gun ion source operated at an accelerating voltage of -6 kV applied to the gun to form the Cs^+ beam. Samples were dissolved in ethylene dichloride and evaporated onto 2-3 μl of glycerol containing 1-2% of thioglycerol as carrier. Spectra were acquired with a SUN data system.

2.8 Preparation of Multilamellar Lipid Bilayers

Water was added to the vacuum dried lipid (1 ml/2 mg lipid). The suspension was then vortexed and frozen in liquid N_2 prior to lyophilization overnight to remove any residual solvent. Multilamellar liposomes were then generated by resuspending the dried lipid in 18 μl of 50 mM Tris-HCl in D_2O , pH 7.05, followed by vortexing. To ensure homogeneity of the liposomes, the multilamellar lipid bilayers were put through four to five freeze-thaw cycles at -70 to 85°C.

2.9 FTIR

For spectroscopic analysis, typically about 5- μl liposome suspension was placed together with powdered α -quartz in a well (0.37 mm diameter) of a stainless steel gasket (0.23 mm thick) that was mounted on a diamond anvil cell at 28°C, as described previously

(Wong et al., 1985). A Digilab FTS-40A Fourier-transform infrared spectrophotometer with a liquid N₂ cooled mercury cadmium telluride detector was used to measure the infrared spectra at 28°C. The infrared beam was focused onto the diamond anvil cell by a sodium chloride lens system. For each spectrum, 512 scans were co-added at a spectral resolution of 4 cm⁻¹. Infrared spectra of hydrated samples of SGC+DMPC (2:3, molar ratio) and SGC+deuterated DMPC (2:3, molar ratio) were recorded as a function of pressure from ambient pressure to 13.2 kbar. The 695 cm⁻¹ phonon band of the α -quartz (internal pressure calibrant) was used to determine the hydrostatic pressure in the gasket of the diamond anvil (Wong et al., 1985). Dry films were prepared by placing a drop of the lipid solution (in CHCl₃) on a CaF₂ window and allowing the solution to dry, followed by purging with a N₂ stream, a 6- μ m spacer was placed between the two CaF₂ windows in the transmission cell. Thermotropic studies were done on the Digilab FTS-40A Fourier-transform infrared spectrophotometer, using a 6- μ l liposome drop placed between two CaF₂ windows separated by a 6- μ m spacer in the transmission cell. A total of 170 scans were co-added at a spectral resolution of 2 cm⁻¹. Data processing was performed using GplotC and RAMOP programs, developed at the National Research Council, Ottawa, ON, Canada (Moffatt et al., 1986). Fourier deconvolution was used to separate unresolved infrared band contours in the original spectra. Fourier derivation was used to determine maximal absorption frequencies in the original spectra.

CHAPTER THREE

RESULTS

3.1 Biochemical Characterization of SGC and SGG

The bovine brain SGC purchased from Sigma and the SGG isolated from ram testes were verified for purity through TLC analysis. Furthermore, the acyl chain identity of SGC subclasses was determined by GLC analysis. The structure of isolated SGG was determined and confirmed to match the known structure through NMR, IR and FAB-MS analysis.

3.1.1 Thin Layer Chromatographic Analysis of SGC and SGG

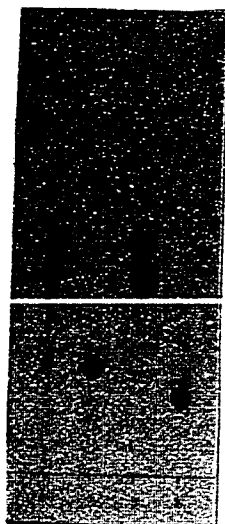
Purity of SGG and SGC samples was verified by TLC using the solvent system (CHCl_3 :MeOH:H₂O, 65:25:4, v/v/v) (Ishizuka et al., 1973) and sugar staining with α -naphthol/H₂SO₄ (Fig. 3.1 and 3.2). Sulfate staining with Azure A/H₂SO₄ was used to verify the presence of the sulfate group in SGG (see Appendix I) (Kates, 1986). The SGG samples showed a single positive α -naphthol staining spot and the SGC sample gave separate spots for α -hydroxy and non-hydroxy species. Upon further heating no other spots (i.e., impurities) were observed in the glycolipid lanes. These results verified that the samples were pure glycolipids. Furthermore, SGG also stained positive as a blue-white spot with the Azure A stain which is specific for sulfate groups and accordingly GG did not stain positive (Fig. I).

Ram testes total lipids revealed a faint glycolipid spot with an R_f value of 0.28 corresponding to the SGG TLC standard (ammonium salt form SGG that was isolated and purified based on SGG standard obtained from Tupper et al., 1994) (reported R_f = 0.30, Ishizuka et al., 1973) (Fig. 3.1, lane 1 and 2, respectively). Lane 3 shows a doublet for GC,

the lower spot with R_f value of 0.48 for α -hydroxy GC and an upper spot with R_f value of 0.53 for non-hydroxy GC (Fig. 3.1). SGC lane 4 showed two spots with R_f values of 0.16 and 0.25, the lower spot representing α -hydroxy SGC and the upper spot representing non-hydroxy SGC (Fig. 3.1).

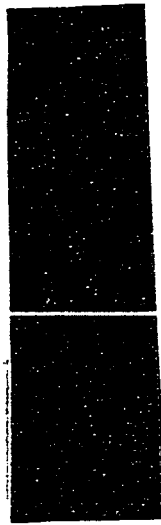
The sodium salt SGG prepared for this study (lane 1, Fig. 3.2) and the ammonium salt of SGG used as a TLC standard (lane 2, Fig. 3.2) gave an R_f value of 0.28. GG gave a single spot in lane 3 with an R_f of 0.70.

Figure 3.1 Thin layer chromatogram: total ram testes extracted lipids (lane 1); standard SGG ammonium (lane 2); GC, α -hydroxy GC (upper spot) and non-hydroxy GC (lower spot) (lane 3); and SGC (used in FTIR studies described in section 3.2) (lane 4). The lipids were separated using the solvent system CHCl_3 :MeOH:H₂O (65:25:4, v/v/v). The lipids were then visualized by α -naphthol/H₂SO₄ staining followed by charring.



1 2 3 4

Figure 3.2 Thin layer chromatogram: SGG sodium salt (prepared for FTIR studies described in section 3.2) (lane 1); standard SGG ammonium salt (lane 2); and GG (lane 3). The lipids were separated using the solvent system $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (65:25:4, v/v/v). The lipids were then visualized by α -naphthol/ H_2SO_4 staining followed by charring.



1 2 3

3.1.2 Gas-Liquid Chromatographic Analysis of SGC

Bovine brain SGC purchased from Sigma contained α -hydroxy and non-hydroxy fatty acid containing subclasses. However, the identity of the molecular species of acyl chains was unknown. The purpose of this study was to characterize the interaction between the symmetric short chain (i.e., C14:0) phospholipid, DMPC, and SGC. In order to discuss possible packing arrangements between these two lipids, it was necessary to determine the acyl chain identity of SGC. The commercial SGC product used here was analyzed by GLC for non-hydroxy and α -hydroxy acids as described in section 2.2. The non-hydroxy acids were analyzed as FAMES and the hydroxy acids as methoxy or acetoxy FAMES (Table 1). The major molecular species present in non-hydroxy SGC subclass were C24:1 (51.5 %), C24:0 (14.5 %), C25:1 (9.8 %) and C26:1 (6.2 %) (weight % of the total non-hydroxy subclass) with lower proportions of C16:0, C18:0, C18:1, C22:0 and C23:0 (Table 1). The major molecular species in the α -hydroxy SGC subclass were C24:1-h (49.7 %), C24:0-h (14.9 %) and C26:1-h (14.3 %) (weight % of the total hydroxy subclass) with lower amounts of C22:0-h, 23:0-h, C23:1-h and C25:1-h (Table 1). The weight ratio of hydroxy to non-hydroxy species was calculated to be 1:2.

Table 1

Fatty Acid Composition of Bovine Brain SGC.^a

Fatty Acid	Non-hydroxy, %	Hydroxy, %
C12:0-15:0	tr.	-
C16:0	1.2	-
C16:1	0.4	-
C18:0	3.9	tr. ^b
C18:1	1.8	-
C18:2	0.4	-
C20:0	0.3	-
C20:1	0.3	-
C22:0	1.4	1.8
C23:0	1.9	1.3
C23:1	-	1.4
C24:0	9.9	4.7
C24:1	35.2	15.8
C25:1	6.7	2.4
C26:1	4.2	4.5

^a Results are given for FAMES derived from total SGC hydroxy and non-hydroxy species after subjection to acetylation treatment, using an SP-2330 column, at 230°C; results are expressed as percent by weight (peak area %) of the total FAMES. Analyses were also performed on unacetylated FAMES at 210°C; any long-chain hydroxy FAMES would not emerge from the column. The latter results, obtained in three separate analyses (not shown here), were consistent with non-hydroxy FAME values of the acetylated sample shown in the table. The weight ratio of hydroxy to non-hydroxy SGC was found to be 1:2.

^b Traces (tr.) of 18:0-h (< 1 %) were present but could not be reliably estimated since the 18:0-acetoxy FAME peak overlapped with that of 24:0 FAME. The presence of 18:0-h was confirmed by the analysis of the methoxylated FAMES (data not shown). Traces (< 0.2 %) of 12:0, 13:0, 14:0 and 15:0 acids were also present.

3.1.3 ^1H and ^{13}C NMR Analysis of SGG

^1H -NMR determines the number of each type of hydrogen in a molecule and the immediate environment of each of these hydrogens (Pavia et al., 1979). Apart from the sugar and glycerol proton resonances in the region of 3-4 ppm, assignments of proton shifts could be made based on the one dimensional (1D) ^1H -NMR spectrum alone. Figure 3.3 presents the 1D ^1H -NMR spectrum of SGG. An alkyl methyl triplet (assigned 6 protons) was observed in the up-field alkyl region at 0.86 ppm, corresponding to the terminal methyl groups of the alkyl ether and fatty acid chain. A 52 proton multiplet was observed at 1.24 ppm, corresponding to methylene ω -1 to acyl C-4 CH_2 groups. At 1.51 ppm and 1.58 ppm two proton multiplets, accounting for four protons, were found which correspond to β -H's to the ether oxygen and carbonyl ester oxygen, respectively. At 2.31 ppm a 2 proton triplet was observed which is characteristic of α -H's to a carbonyl ester bond. A 2 proton multiplet was observed at $\sim$ 3.39 ppm, corresponding to α -H's to an ether bond. At 4.39 a 3 proton signal was observed that likely reflects the resonances of the β anomeric H and other proton resonances. At 2.75 ppm a broad resonance was located corresponding to ammonium protons. Another broad signal was found at 6.55 ppm that was likely due to sugar hydroxy proton resonance.

The down-field region contains proton resonances resulting from glycerol moiety protons and sugar moiety protons (Alvarez et al., 1990). The two dimensional heteronuclear multiple quantum coherence (2D-HMQC) NMR spectrum (i.e., ^1H - ^{13}C 2D-NMR) was used to assign protons and their respective carbon's chemical shifts in this region. Figure 3.4 presents the 2D HMQC spectrum of SGG. The *sn*-2 glycerol methine H was found to

resonate at 5.2 ppm with a carbon resonance at 71.3 ppm, in good agreement with the proton NMR results of Ishizuka et al. (1973) and Alvarez et al. (1990). The *sn*-1 glycerol CH₂ group was found to possess protons that resonated at 3.77 ppm and a carbon resonance at 68.8 ppm. The *sn*-3 CH₂ group had two different resonances for each proton as described by Alvarez et al. (1990); one set of proton and carbon resonances were found at 3.78 ppm and 60.0 ppm, respectively, and the other set 3.80 ppm and 62.5 ppm, respectively. The sugar group resonances were also assigned based on the 2D-HMQC spectrum. The proton and carbon at position 2 (C-2) of the galactose have resonances at 3.55 ppm and 68.8 ppm, respectively. The proton and carbon shifts of the atoms at C-5 of the sugar were found to be at 3.60 and 71.0 ppm, respectively. C-6 proton and carbon possessed chemical shifts at 3.60 and 73.0 ppm, respectively. A second set of shifts attributable to the second proton at C-6 was 3.77 ppm for the proton shift and 72.0 ppm for the carbon shift, in qualitative agreement with the proton assignments of Alvarez et al. (1990). The proton and carbon resonances for the atoms at C-4 of the sugar were found at 3.93 and 68.8 ppm, respectively. The proton and carbon shifts for the atoms at C-3 were located at 3.97 and 68.8 ppm, respectively.

The number of protons found by peak integration of the ¹H-NMR spectrum corresponding to SGG was 80.3 while the number of protons calculated based on the known structure of the free acid form with C16:0 acyl and alkyl chains (Ishizuka et al., 1973; Alvarez et al., 1990; Tupper et al., 1994) was 79. The NMR results obtained for the SGG isolated and purified for the FTIR studies described in section 3.2 indicated that the structure of the lipid isolated by adsorption column chromatography corresponded to the lipid described as a monoalkylmonoacyl-3'-sulfogalactosylglycerolipid (see Fig. 1.2). Table 2

summarizes all NMR results for purified ram testes SGG.

Table 2

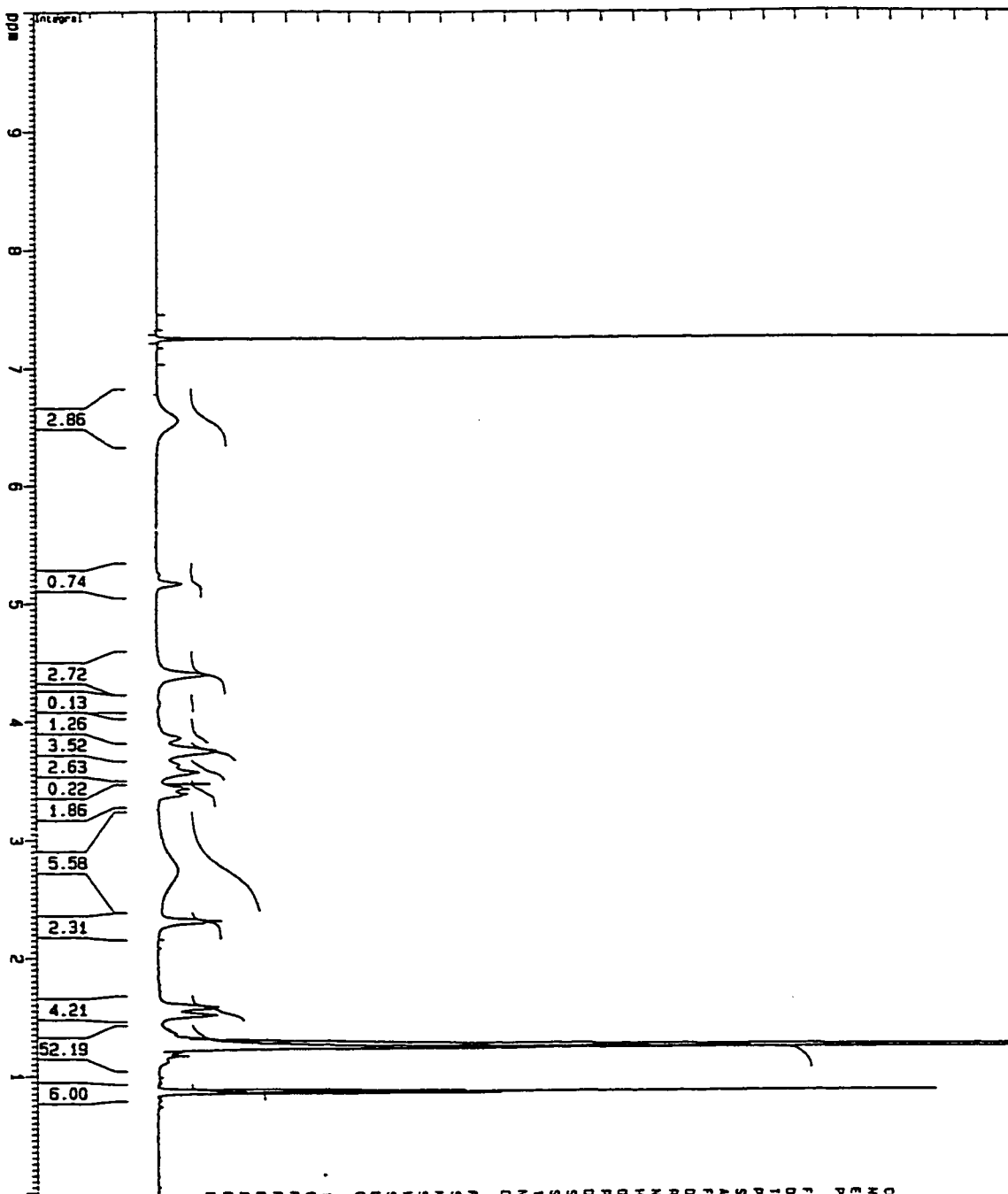
¹H-NMR and ¹³C-NMR results for purified ram testes SGG

Group Identity	δ_H (ppm)	Number of Protons: ^a		δ_C (ppm)
		Found	Calculated	
terminal CH ₃	0.86	6.00	6	14.0
(ω -1) methylene CH ₂	1.24	52.19	50	22.6
acyl C4 - C(ω -3) methylene	1.24			30.0
(ω -2) methylene CH ₂	1.24			32.0
β -CH ₂ to ether O	1.51	4.21	2	30.0
β -CH ₂ to ester carbonyl	1.58		2	24.9
α -CH ₂ to ester carbonyl	2.31	2.31	2	34.5
α -CH ₂ to ether	3.39	1.86	2	71.0
C2 of sugar	3.55	2.63	1	68.8
C5 of sugar	3.60		1	71.0
C6 of sugar	3.60		1	73.0
<i>sn</i> -1 glycerol methylene CH ₂	3.77	3.52	2	68.8
C6 of sugar	3.77		1	72.0
<i>sn</i> -3 methylene CH ₂ a	3.80		1	60.0
<i>sn</i> -3 methylene CH ₂ b	3.80	1.26	1	62.5
C4 of sugar	3.93		1	68.8
C3 of sugar	3.97	2.72	1	68.8
β anomeric H	4.39		1	- ^b
<i>sn</i> -2 methine CH	5.20	0.74	1	71.3
OH protons	6.55	2.86	3	-
NH protons	2.75	5.58	3	-

^a The first column (i.e., Found) lists the peak integration values from the ¹H-NMR spectrum. The total number of protons found through peak integration of the ¹H-NMR spectrum was 80.3. The second column (i.e., Calculated) lists the values of expected protons based on the known structure. The total number of protons calculated based on the known structure of the free acid with C16:0 acyl and alkyl chain was 79.

^b A ¹³C chemical shift could not be assigned to position C-1 of the sugar.

Figure 3.3 One dimensional 500 MHz proton NMR spectrum of purified ram testes SGG.



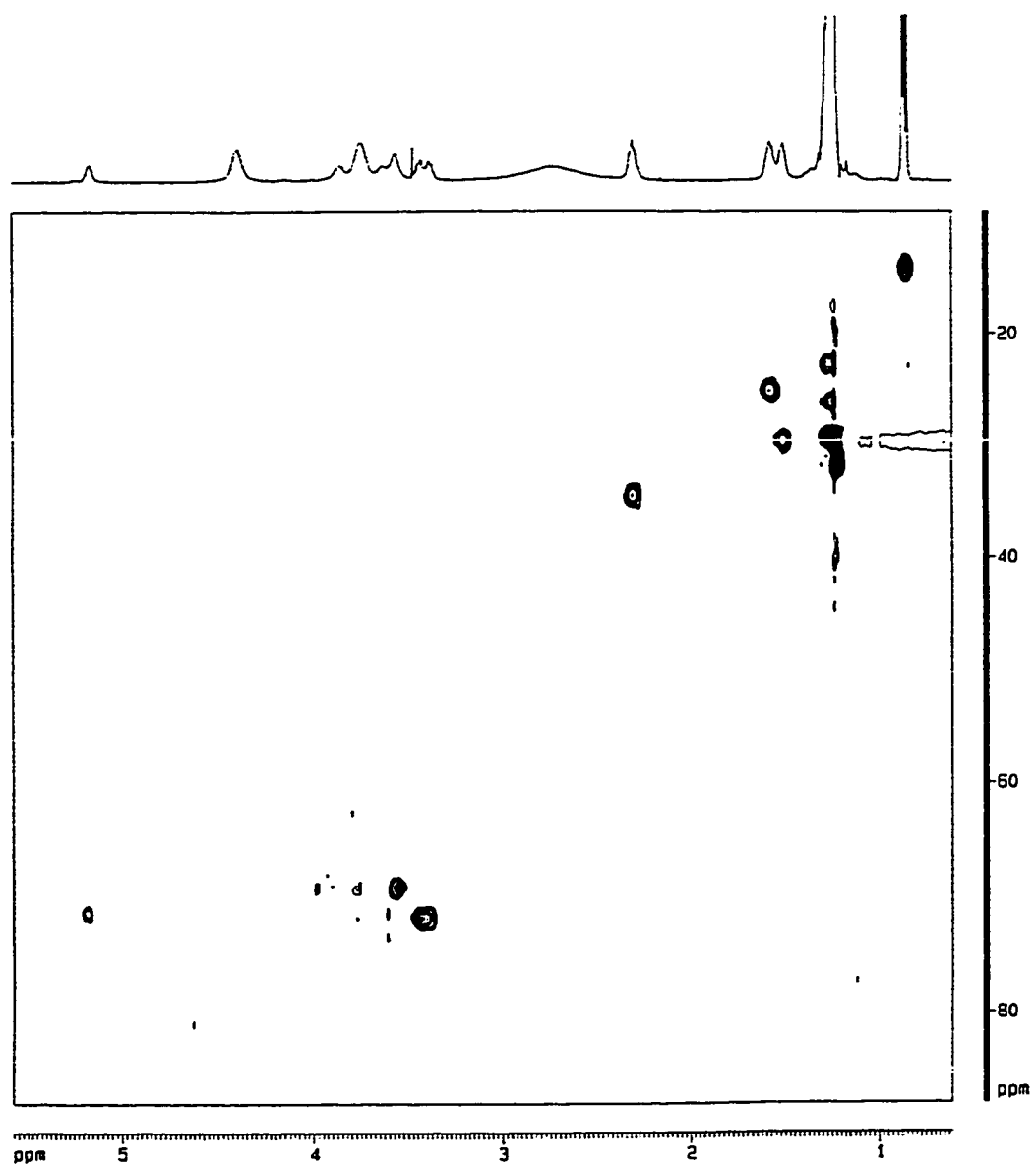
Current Data Parameters
NAME: meta_2
EXPNO: 1
PROCNO: 1

F2 - Acquisition Parameters
Date_ Time: 980209 14.43
PULPROG: zg
SOLVENT: CDCl3
AQ: 4.6538823 sec
FIDRES: 0.107456 Hz
AQ: 71.0 usec
RG: 2048
NUC1: 1H
NUC2: 13C
P1: 0.0100000 sec
DE: 3.0 usec
SF01: 500.1361707 MHz
SMA: 7042.25 Hz
TO: 65336
DS: 32
OS: 0

F1 - Processing parameters
SI: 32768
MC2: OF
SF: 500.1364311 MHz
WDW: EM
SSB: 0
LB: 0.30 Hz
GB: 0

10 user plot parameters
CX: 22.00 cc
FIP: 10.000 ppm
F1: 5001.35 Hz
F2P: 0.000 ppm
F2: 0.00 Hz
PPMCK: 0.45455 ppm
HZCK: 227.33429 MHz

Figure 3.4 Two dimensional heteronuclear multiple quantum coherence (2D-HMQC) spectrum of purified ram testes SGG from the proton region of 0.5-5.5 ppm and carbon region 10-85 ppm.



3.1.4 Fast Atom Bombardment-Mass Spectrometry (FAB-MS) Analysis of SGG

Mass spectrometry subjects molecules to bombardment by a stream of high energy electrons that converts the molecules to ions. The ions are accelerated in an electric field and then separated according to their mass-to-charge (m/z) ratio. The ions with a particular m/z ratio are detected by a device which counts the number of ions which strike it. A mass spectrum is then produced, which is a plot of the number of particles detected as a function of the m/z ratio (Pavia et al., 1979). The data from a mass spectrum can be used to determine the structure of the molecule being studied by examining the major ion peaks. The negative FAB mass spectrum of SGG is presented in Fig. 3.5. The major ion peak at m/z 795 corresponds to deprotonated SGG with a C16:0 acyl and alkyl chain ($C_{41}H_{79}O_{12}S$; $M-H=795$). Ion peaks at m/z 255 and m/z 241 were assigned to each the acyl chain ($C_{16}H_{31}O_2$; $M-H=255$) and the alkyl chain ($C_{16}H_{33}O$; $M-H=241$), respectively. The fragment ion at m/z 299 corresponds to an SGG molecule that has lost both its acyl and alkyl chain ($C_9H_{15}O_9S$; $M=299$). An ion peak for the galactose sulfate group was found at m/z 259 ($C_6H_{11}O_5S$; $M-H=259$). These negative FAB-MS results are summarized in Table 3; and analysis of the minor molecular species are summarized in Appendix II. These results are in good agreement with the FAB-MS results reported by Ishizuka et al. (1973) and Alvarez et al (1990). As well, the structure determined and molecular subclasses identified for the SGG isolated and purified from ram testes for this study corresponds with the acyl and alkyl chain identities of ram testes SGG reported by Tupper et al. (1994).

Table 3

Negative FAB-MS major ion peaks of purified ram testes SGG with C16:0 acyl and alkyl chains. ^a

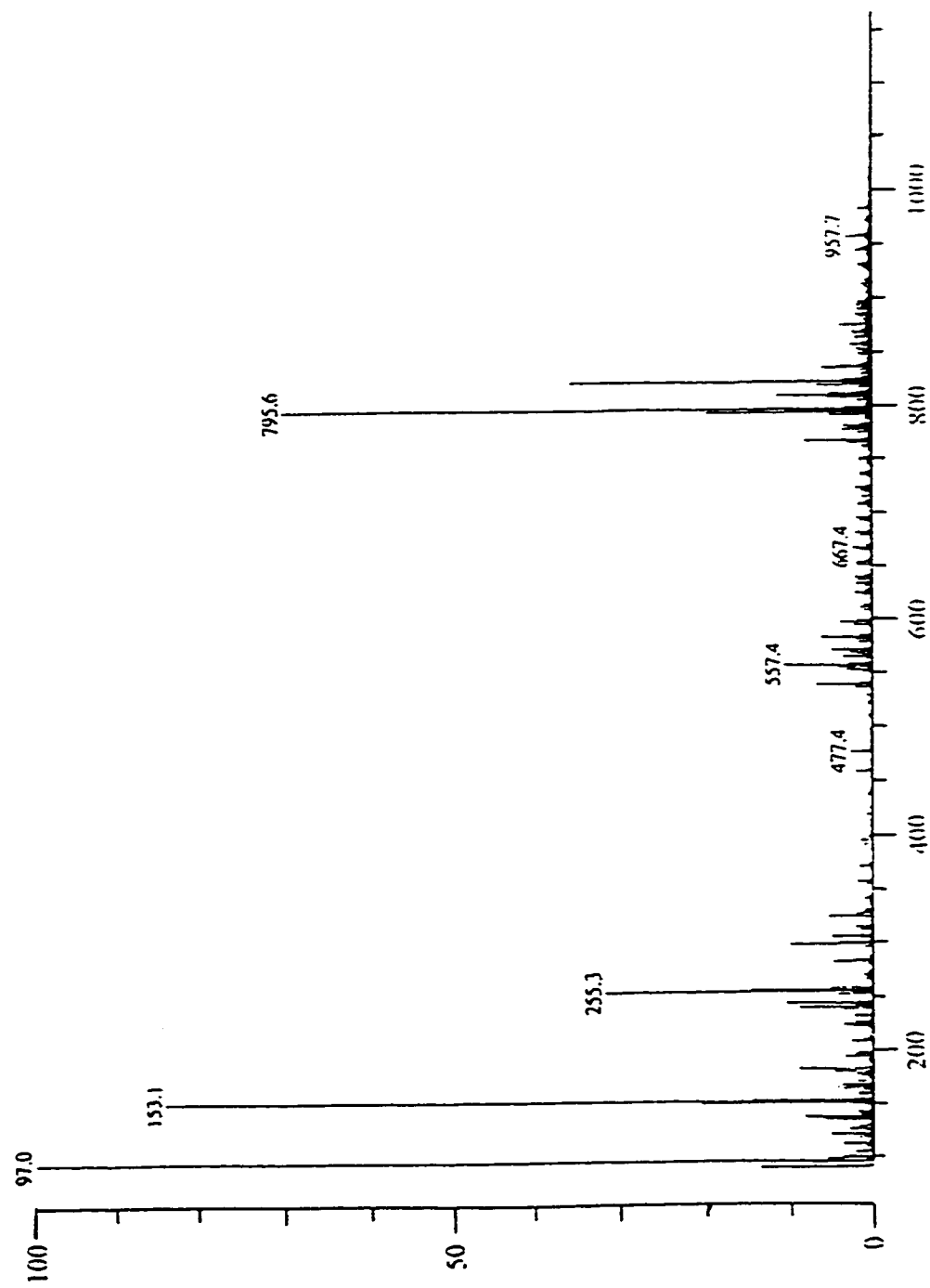
Identity of Ion Peaks	Mass ^b		Relative Abundance(%) ^c
	Found	Calculated	
SGG (16:0/16:0)	795.6	795	70.0
SGG without <i>sn</i> -2 acyl chain	539.4	539	6.9
SGG without <i>sn</i> -2 and <i>sn</i> -1 chains	299.1	299	10.1
galactose sulfate group	259.1	259	5.2
<i>sn</i> -2 C16:0 fatty acid	255.3	255	31.8
<i>sn</i> -1 C16:0 alcohol	241.1	241	9.0

^a Other peaks were identified in the mass spectrum corresponding to SGG molecular species that were found to be minor components (Tupper et al., 1994). The negative FAB-MS results for these minor components are summarized in Table I in appendix II.

^b The Mass value in the first column (i.e., Found) represents the ratio of the mass of the ion to the charge on the ion (Pavia et al., 1979).

^c This value represents the percent relative abundance of a specific ion peak relative to the abundance of the base ion peak (corresponds to SO₃⁻NH₃ with m/z of 97.0). This percent value reflects the stability of the molecular ion formed rather than the relative amount formed of the ion.

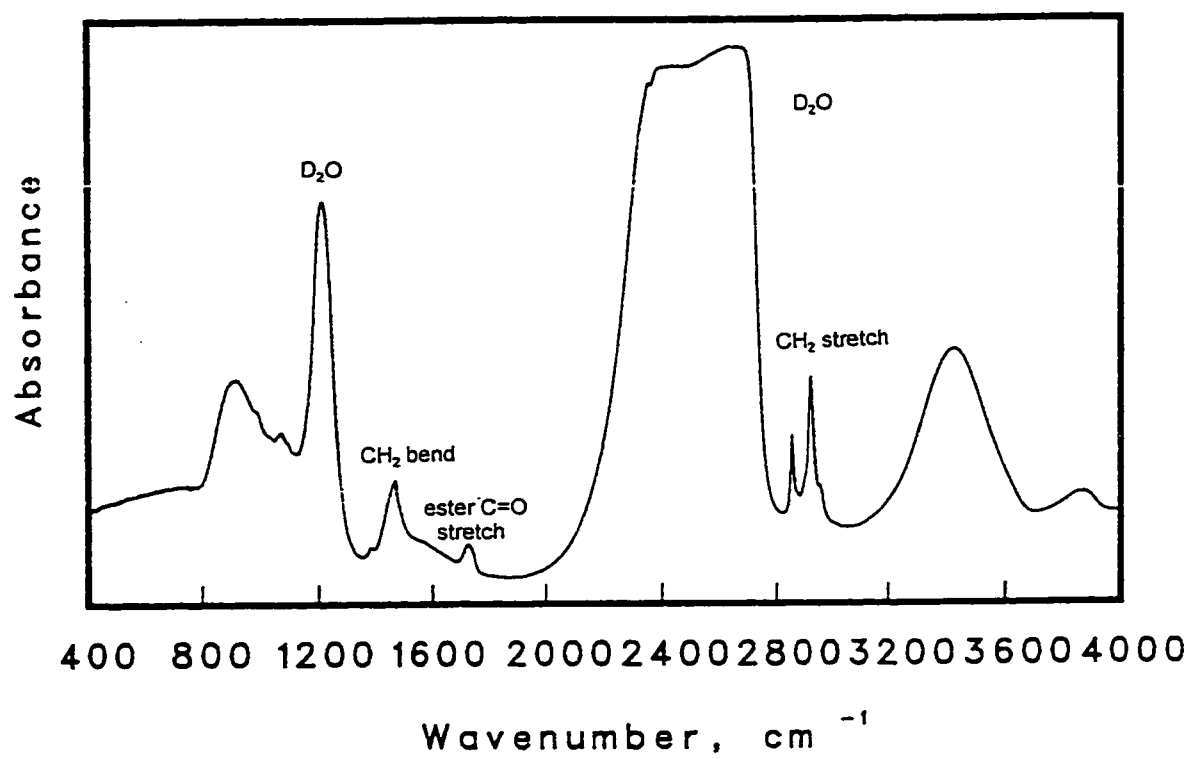
Figure 3.5 Negative FAB-MS spectrum of purified ram testes SGG.



3.1.5 Infrared Spectroscopic Characterization of SGG

SGG extracted and purified from ram testes could be partially characterized by examining its IR spectrum to verify the intactness of the interfacial region and hydrocarbon region (Fig. 3.6). At $\sim 1467\text{ cm}^{-1}$ the CH_2 bending vibration was observed. The carbonyl was found to absorb at $\sim 1735\text{ cm}^{-1}$. The symmetric and antisymmetric CH_2 groups were found to absorb at $\sim 2850\text{ cm}^{-1}$ and 2920 cm^{-1} , respectively.

Figure 3.6 Infrared spectrum of purified ram testes SGG in the frequency range 400-4000 cm^{-1} . The lipid sample was hydrated in 50 mM Tris-HCl in D_2O and FTIR spectral data was collected at 28°C.

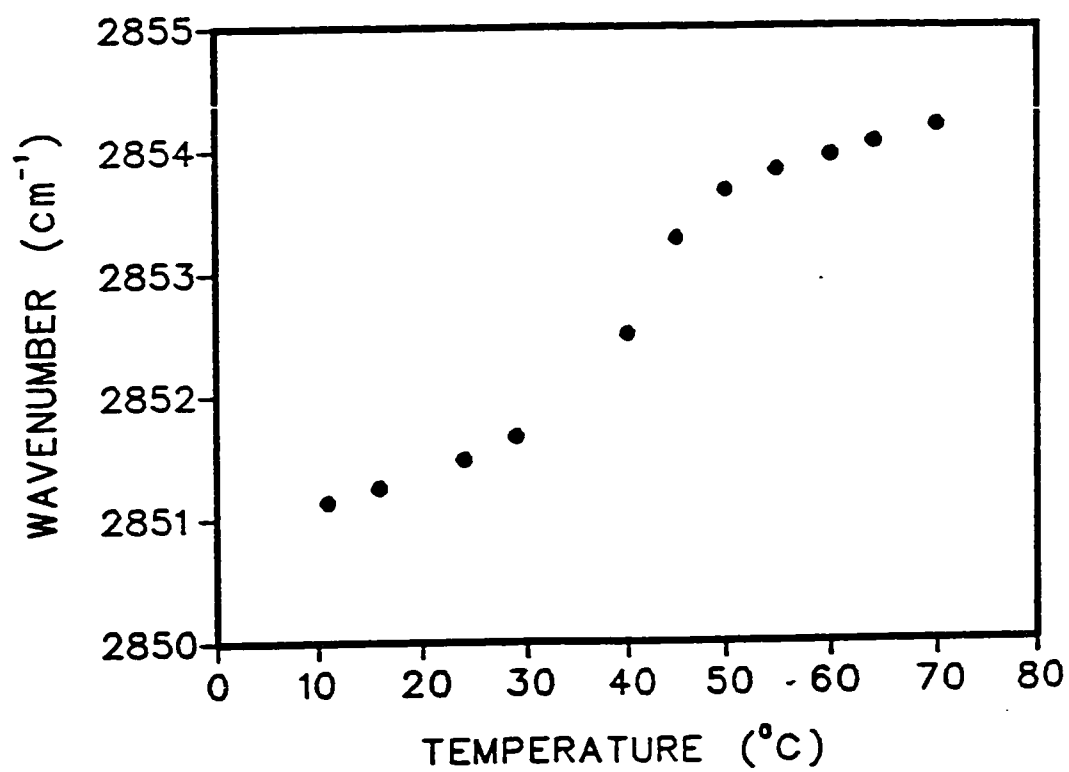


3.2 FTIR Analysis of SGC and SGG

3.2.1 Thermotropic Analysis of SGC+DMPC

In order to determine the physical state of our mixed model system (SGC+DMPC) at the FTIR operating temperature of 28°C, as well as establish if the two lipids were indeed interacting, the T_m of the mixed bilayers was determined. Figure 3.7 illustrates the FTIR temperature profile of mixed lipid liposomes of SGC and DMPC (molar ratio 2:3) in the CH_2 symmetric stretching region of 2850 to 2855 cm^{-1} . The T_m of the transition from the gel-to-liquid crystalline phase of SGC+DMPC liposomes was at 42°C. The transition started at 30°C and was complete at 50°C. The T_m of DMPC has been determined to be 23.6°C by thermotropic FTIR in the laboratory of Dr. Danielle Carrier, in which we performed all our thermotropic analyses and the T_m of synthetic C24:1 SGC has been reported to be 50.3°C (Boggs et al., 1988), or 61-80°C for other components of the SGC commercial sample (Boggs et al., 1993).

Figure 3.7 FTIR temperature profile of mixed lipid liposomes consisting of SGC and DMPC (molar ratio 2:3). The wavenumber of the CH₂ symmetric stretching mode (2850-2855 cm⁻¹) was recorded with increasing temperature. The lipid sample was hydrated in Tris-HCl in D₂O.



3.2.2 Hydrogen Bonding Studies at the Interfacial Region of SGC+DMPC liposomes

Examination of the absorption patterns of the amide C=O of SGC and the ester C=O of DMPC would elucidate the nature of the interaction, at the interfacial region, between these two lipids in mixed bilayers (SGC+DMPC, 2:3 molar ratio). Shifts in these stretching frequencies in mixed liposomes, as compared to that in single lipid liposomes, can be used to monitor changes in interfacial hydrogen bonding.

Figure 3.8A illustrates the absorption of the amide I C=O bond of SGC and of SGC in mixed liposomes with DMPC (2:3 molar ratio). The broad amide I band arising from SGC in the mixed liposomes shifted to a frequency of 1645 cm^{-1} compared to a frequency of 1624 cm^{-1} for the SGC liposomes (Fig. 3.8A). The Fourier deconvolved spectra of the same region are presented in Figure 3.4B. The low frequency component of the amide I group of SGC was shifted from a frequency of 1619 cm^{-1} in the SGC liposomes to 1626 cm^{-1} in the mixed liposomes. The higher frequency component (1640 cm^{-1}) of SGC shifted to a frequency of 1648 cm^{-1} in the mixed liposomes. Furthermore, the intensity of the high frequency component increased concomitantly with a decrease in the intensity of the low frequency component.

Overall, these results indicate that the insertion of DMPC into SGC bilayers has resulted in an increase in double-bond character of the amide I C=O groups of SGC. This may be a consequence of a shielding effect of DMPC on SGC such that intermolecular hydrogen bonding between SGC molecules is disrupted.

Figure 3.8 (A) Infrared spectra in the amide I stretching band region ($1600\text{--}1670\text{ cm}^{-1}$) of the SGC liposomes (---) and the mixed lipid liposomes of SGC + DMPC (molar ratio 2:3) (- - -). (B) The Fourier deconvolution of spectra in (A). A breakpoint of 1.4 was used in the Fourier domain for deconvolution. The spectra were plotted in autoscale. Lipid samples were hydrated in 50 mM Tris-HCl in D_2O and spectral data was collected at an FTIR operating temperature of 28°C .

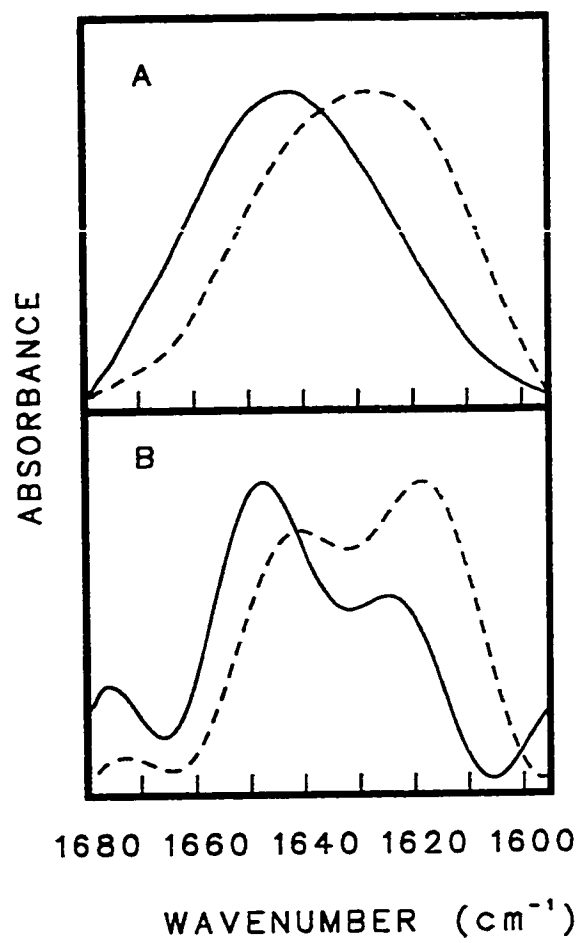
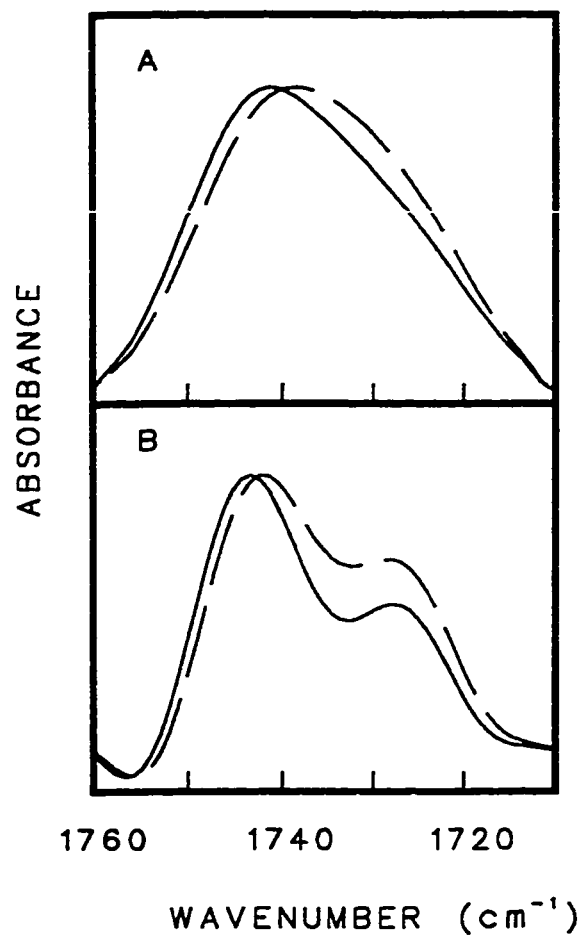


Figure 3.9 illustrates the absorption of the ester C=O band of DMPC and of DMPC in mixed lipid liposomes with SGC. The broad band arising from the ester C=O of DMPC in the mixture with SGC was shifted slightly to a higher frequency (1741 cm^{-1}) as compared to the DMPC liposomes (1738 cm^{-1}) (Fig. 3.9A). The Fourier deconvolved spectra of the same region are presented in Figure 3.9B. The low frequency component of the ester C=O group of DMPC in mixed liposomes with SGC did not show a significant shift when compared to pure DMPC liposomes. The higher frequency component (1741 cm^{-1}) of DMPC shifted to a frequency of 1743 cm^{-1} in the mixed lipid liposomes (Fig. 3.9B). The higher frequency component of the DMPC liposomes and the DMPC+SGC liposomes were normalized to the same intensity. The intensity ratio of the lower frequency component relative to the higher frequency was decreased in the mixed liposomes, as compared to the ratio observed for the DMPC liposomes.

SGC insertion into DMPC bilayers has resulted in a decrease in the transition moment of the ester C=O groups of DMPC absorbing at lower frequency as indicated by the decrease in intensity ratio of the lower frequency component to the higher frequency component. This indicated that the ester C=O groups of DMPC in mixed lipid liposomes absorbing at lower frequency had a reduced hydrogen bonding potential in mixed liposomes which likely resulted in a disruption of the interfacial hydrogen bonding network in mixed liposomes.

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Fig. 3.9 (A) Infrared spectra of the ester C=O stretching region ($1680\text{--}1780\text{ cm}^{-1}$) of the DMPC liposomes (— —) and the mixed lipid liposomes of SGC+DMPC (molar ratio 2:3) (——). (B) The Fourier deconvolution of spectra in (A). A breakpoint of 1.6 was used in the Fourier domain for deconvolution. The spectra were plotted in autoscale. Lipid samples were hydrated in 50 mM Tris-HCl in D_2O and spectral data was collected at an FTIR operating temperature of 28°C .



3.2.3 Hydrocarbon Chain Region of SGC+DMPC

The order/disorder and packing of hydrocarbon chains will determine the fluidity of bilayer membranes. This barotropic study was conducted to determine the orientational disorder introduced into the hydrocarbon chains of bilayer membranes upon interaction between SGC and DMPC in the mixed liposomes. Figure 3.10 presents the pressure related stacked contour plots of the CH_2 bending mode of the SGC+deuterated DMPC bilayers. The use of deuterated DMPC in the liposome mixture resulted in a shift of the absorption bands arising from DMPC hydrocarbon chains to a higher frequency, so that the observed CH_2 bending mode spectrum represented that of SGC hydrocarbon chains. A broadening of this bending mode band, which was centered at $\sim 1467 \text{ cm}^{-1}$ at ambient pressure, was observed at 9.2 kbar and continued with increasing pressure up to 13.2 kbar. The expected correlation field splitting of this vibrational mode was not observed with increasing pressure. In order to better understand the dynamics of the hydrocarbon chains in this mixture of SGC and deuterated DMPC, a pressure dependence plot was calculated. Figure 3.11 presents the pressure dependence plot of the frequencies of the CH_2 bending mode for mixed liposomes of SGC+deuterated DMPC. The pressure dependence plot was based on the calculation of maximal CH_2 bending frequencies with increasing pressure from the third order Fourier derivative spectra. This pressure dependence plot reveals the onset of a correlation field splitting at 7.7 kbar with two separate frequency components first appearing at 9.2 kbar.

Figure 3.12 presents the stacked contour plots of the methylene bending mode region of mixed liposomes of SGC + DMPC (not deuterated). The spectra reflect contributions from both DMPC hydrocarbon chains and SGC hydrocarbon chains. Inspection of the

stacked contour plots of SGC+DMPC liposomes reveals the onset of a correlation field splitting at 5.3 kbar with two separate frequency peaks gradually becoming more visible on increasing pressure up to 12.6 kbar.

Figure 3.13 displays the pressure dependence plot of the CH_2 bending mode frequencies and reveals an onset of correlation field splitting at ~ 4 kbar, with the initial appearance of an observable shoulder at the high frequency side of the CH_2 bending vibration at 5.3 kbar

Correlation field splitting of the CH_2 bending mode of SGC bilayers occurs at 5.1 kbar (Tupper et al., 1992) and at 3.2 kbar for DMPC bilayers (Auger et al., 1988; Wong et al., 1988; Wong, 1994). The liposome systems examined in this study did not split at these reported values. Thus, the two lipids, SGC and DMPC, indeed are interacting such that the barotropic behaviour of the lipids was affected in the mixed liposomes, leading to an increase in disorder of the hydrocarbon region.

Figure 3.10 Stacked contour plots, as a function of pressure, of the infrared spectra of the CH_2 bending mode ($1420\text{-}1500\text{ cm}^{-1}$) of the mixed liposomes of SGC+deuterated DMPC. The numbers to the right of the contour plots are the pressure values in kbar. The lipid sample was hydrated in 50 mM Tris-HCl in D_2O and spectral data was collected at an FTIR operating temperature of 28°C .

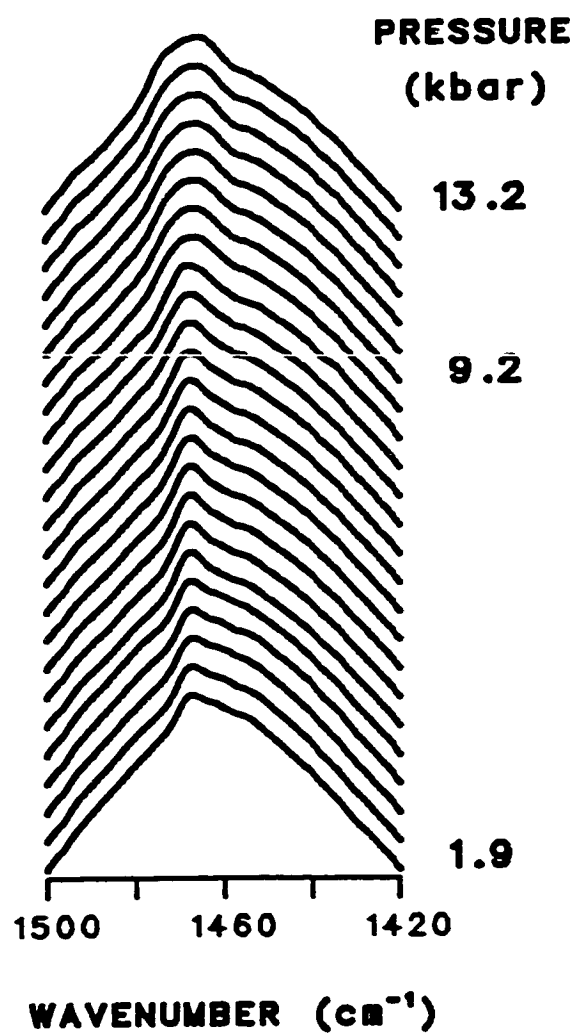


Figure 3.11 Pressure dependence of the frequencies of the CH₂ bending mode (1458-1476 cm⁻¹) for the mixed liposomes of SGC+deuterated DMPC. A breakpoint of 0.95 was used in the Fourier domain for derivatization.

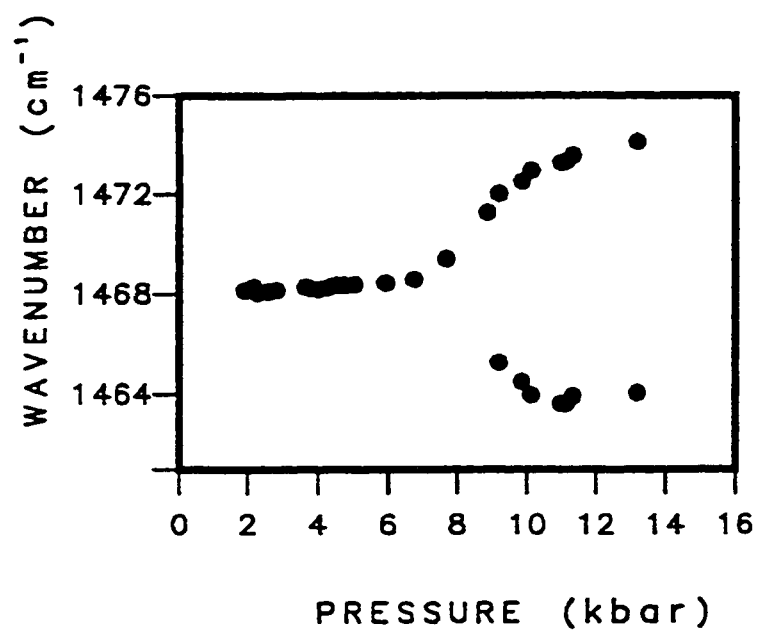


Figure 3.12 Stacked contour plots, as a function of pressure, of the infrared spectra of the CH_2 bending mode ($1420\text{--}1500\text{ cm}^{-1}$) of the mixed liposomes consisting of SGC+non-deuterated DMPC. The numbers to the right of the contour plots are the pressure values in kbar. The lipid sample was hydrated in 50 mM Tris-HCl in D_2O and spectral data was collected at an FTIR operating temperature of 28°C .

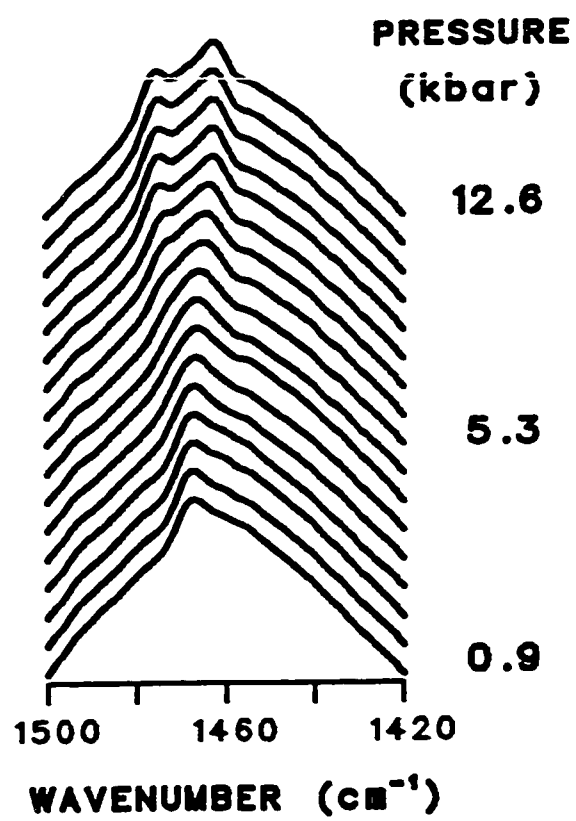
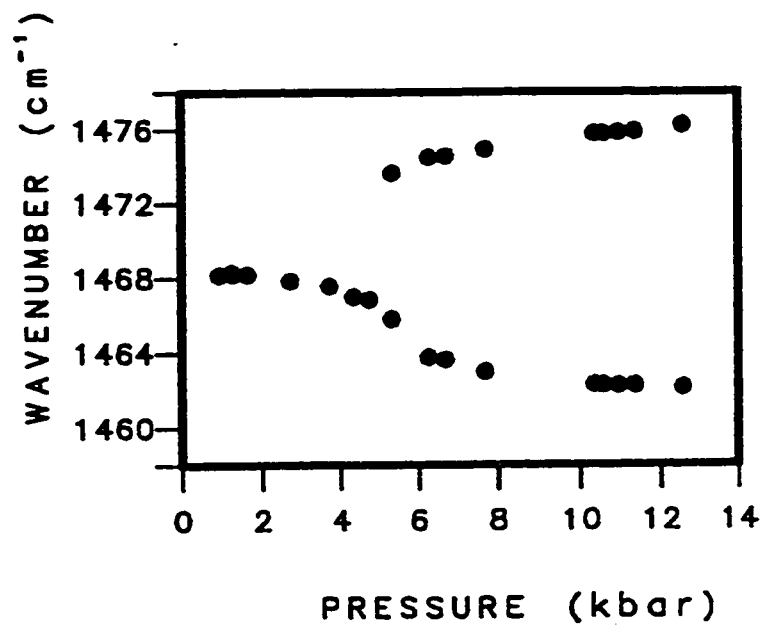


Figure 3.13 Pressure dependence of the frequencies of the CH_2 bending mode ($1458\text{-}1476\text{ cm}^{-1}$) for the mixed liposomes consisting of SGC+non-deuterated DMPC. A breakpoint of 0.95 was used in the Fourier domain for derivatization.



3.2.4 Thermotropic Analysis of SGG and SGG+DMPC

Figure 3.14 illustrates the FTIR temperature profiles (revealing the gel-to-liquid crystalline phase transition) of SGG liposomes, DMPC liposomes and SGG+DMPC (molar ratio 2:3) mixed liposomes in the wavenumber range of 2849 to 2855 cm^{-1} (the CH_2 symmetric stretching band region). While the T_m of DMPC was known to be 23.6°C (Bou Khalil and Carrier, personal communication), the T_m of SGG or SGG+DMPC liposomes has never been reported in the literature. Our studies revealed the T_m of SGG and SGG+DMPC mixed liposomes (2:3 molar ratio) were at 45°C and 28°C, respectively (Fig. 3.14).

At 5°C, the first temperature at which CH_2 symmetric stretching was monitored, it was observed that the CH_2 absorption frequency of DMPC (2849.6 cm^{-1}) was the lowest followed by SGG liposomes (2850.2 cm^{-1}), and finally SGG+DMPC mixed liposomes (2850.5 cm^{-1}) which absorbed at the highest frequency (Fig. 3.14). Thus, it appeared that DMPC molecules had the least amount of gauche conformers in their acyl chains followed by SGG and finally the mixed liposomes contained the highest number of gauche conformers in their hydrocarbon chains.

Both the temperature profile of DMPC and of SGG+DMPC liposomes showed a single smooth cooperative transition. The onset of transition of DMPC liposomes was at 20°C and was complete at 40°C, whereas the onset of transition of SGG+DMPC mixed liposomes was at 15°C and was complete at 35°C. In contrast, the temperature profile of SGG liposomes indicated that SGG underwent one main transition from the gel-to-liquid crystalline phase, as well as one pretransition. The onset of the main transition was at 41°C and was complete at 50°C. The onset of the pretransition was at 20°C and was complete

upon the onset of the main transition.

The pretransition of SGG liposomes could also be monitored by observing the changes to the ester C=O band of SGG upon increasing temperature (Fig. 3.15). These stacked contour plots illustrated the appearance of two distinct populations of C=O ester groups, one group absorbing at high frequency ($\sim 1739\text{ cm}^{-1}$) and one absorbing at lower frequency ($\sim 1719\text{ cm}^{-1}$). The first detectable appearance of two groups was at 20°C and the two C=O bands were no longer evident at 41°C . It is plausible that at 20°C the SGG molecules began to undergo a pretransition into a molecular arrangement that resulted in two distinct populations of ester C=O groups, one absorbing at high frequency and one absorbing at lower frequency. Furthermore, upon onset of the main transition from the gel-to-liquid crystalline phase these two distinct populations of ester C=O groups no longer existed in the new physical state of the bilayers.

Figure 3.14 FTIR temperature profile of the SGG (■), DMPC (▼) (Data collected in the laboratory of Dr. Danielle Carrier) and mixed liposomes consisting of SGG and DMPC (molar ratio 2:3) (●). The wavenumber of the CH₂ symmetric stretching mode (2849-2855 cm⁻¹) was recorded with increasing temperature. Lipid samples were hydrated in Tris-HCl in D₂O.

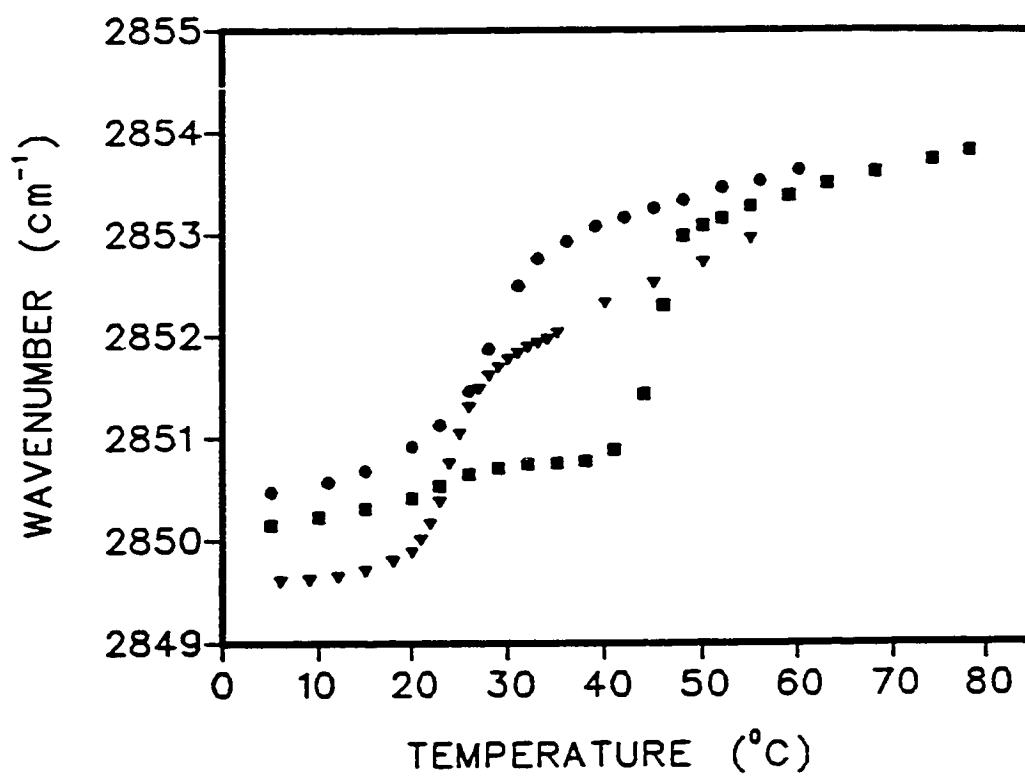
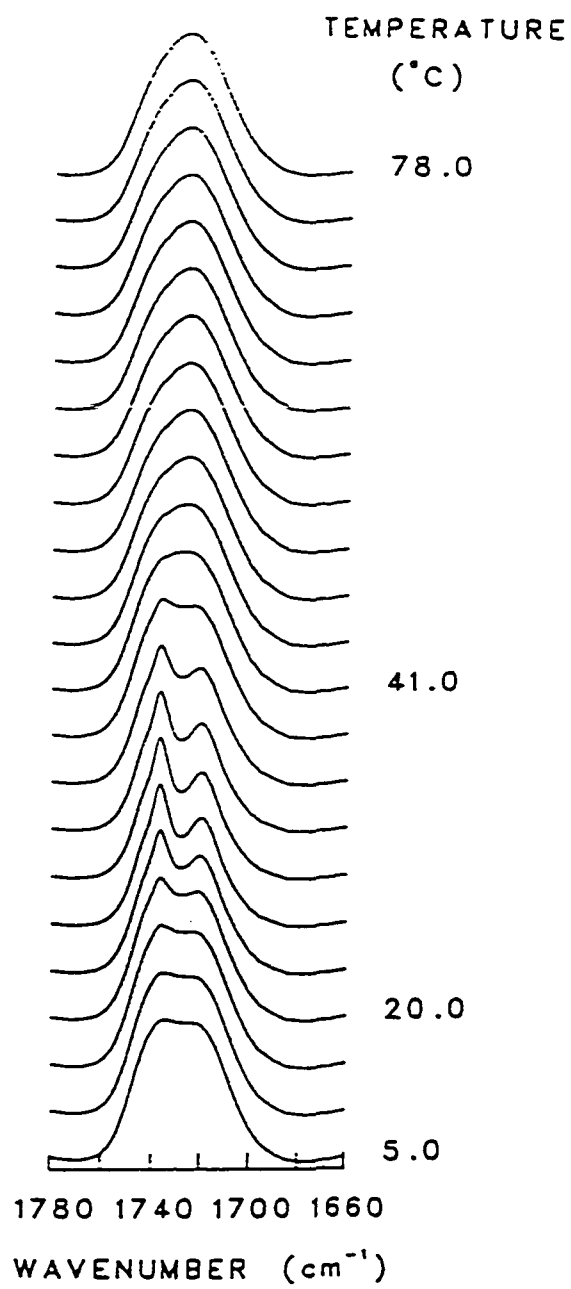


Figure 3.15 Stacked contour plots, as a function of temperature, of the infrared spectra of the C=O stretching band ($1780\text{-}1660\text{ cm}^{-1}$) of SGG liposomes. The numbers to the right of the contour plots are the temperature values. These spectra were original, i.e., were not processed through Fourier deconvolution or derivation manipulations. The lipid sample was hydrated in 50 mM Tris-HCl in D_2O .



3.2.5 Hydrogen Bonding Studies at the Interfacial Region of SGG+DMPC liposomes

Absorption patterns in ester C=O stretching region were examined to study the interactions between SGG and DMPC at the glycerol moiety-acyl chain interface of mixed liposomes. Figure 3.16 illustrates the absorption spectra of the ester C=O bond of SGG liposomes and the combined absorption of the ester C=O bond of SGG and the ester C=O bond of DMPC in the mixed liposomes (2:3 molar ratio) in the gel phase (5°C) (Fig. 3.16A), at physiological temperature (37°C) (Fig. 3.16C) and in the liquid crystalline phase (55°C) (Fig. 3.16E). The Fourier deconvolved spectra of the same region, at the same temperature, are presented to the right of the original spectra in Figures 3.16B, 3.16D and 3.16F. Upon deconvolution of the broad ester bands, two component bands were revealed at all temperatures, one absorbing at a low frequency and one absorbing at higher frequency.

In the gel phase (5°C), the broad ester C=O band of SGG liposomes is wider than that of the mixed SGG+DMPC liposomes, and appears to contain a component absorbing at a lower frequency that is not present in the mixed SGG+DMPC liposomes (Fig. 3.16A). The deconvolved spectra reveal that the high frequency component of the ester C=O band of the mixed liposomes absorbed at a slightly higher frequency (1743 cm⁻¹) than that of SGG liposomes (1741 cm⁻¹) (Fig. 3.16B). As well, the low frequency component of the mixed liposomes (1725 cm⁻¹) absorbed at a higher frequency than the low frequency component of SGG liposomes (1717 cm⁻¹). The intensity ratio of the low frequency component relative to the high frequency component has decreased for mixed SGG+DMPC liposomes as compared to SGG liposomes.

At physiological temperature (37°C), it appears the difference in bandwidth of the

ester C=O band of mixed liposomes relative to SGG liposomes at physiological temperature has decreased compared to the gel phase (Fig. 3.16C). Examination of the Fourier deconvolved spectra of the same region shows a shift down of the high frequency component of SGG liposomes (1737 cm^{-1}) and a shift up of the low frequency component (1720 cm^{-1}) of SGG liposomes. Furthermore, the lower frequency component of the mixed liposomes was found to shift up to 1727 cm^{-1} and the high frequency component absorbed at 1744 cm^{-1} (Fig. 3.16D). The intensity ratio of the lower frequency component to the higher frequency component of mixed liposomes is higher at physiological temperature than in the gel phase at 5°C . Figure 3.16E presents the ester C=O absorption of the mixed liposomes and SGG liposomes at 55°C , in the liquid crystalline phase. The mixed liposomes absorb at a higher frequency relative to SGG liposomes. The Fourier deconvolved spectra show the higher frequency component of the mixed liposomes absorbed at 1744 cm^{-1} and the higher frequency component of SGG liposomes absorbed at 1743 cm^{-1} (Fig. 3.16F). The lower frequency component of the mixed liposomes absorbed at 1728 cm^{-1} and lower frequency component of SGG liposomes absorbed at 1722 cm^{-1} . Furthermore, the relative intensity of the high frequency component of SGG liposomes decreased with a concomitant increase in the lower frequency component of SGG liposomes.

In general, the shifts to higher frequencies of the mixed liposomes as compared to SGG liposomes, at all temperatures, indicates the ester C=O groups of mixed liposomes possess more double-bond character. Thus, it appears the insertion of DMPC into SGG bilayers, at all temperatures, results in a disruption of hydrogen bonding between SGG molecules.

Figure 3.16 Infrared spectra of the ester C=O stretching region ($1760\text{--}1680\text{ cm}^{-1}$) of SGG liposomes (---) and SGG+DMPC (2:3 molar ratio) (—) at 5°C , in the gel phase (A); at 37°C , physiological temperature (C); and at 55°C , in the liquid crystalline phase (E). (B), (D), and (F) are the Fourier deconvolved spectra of (A), (C), and (E). Lipid samples were hydrated in 50 mM Tris-HCl in D_2O .

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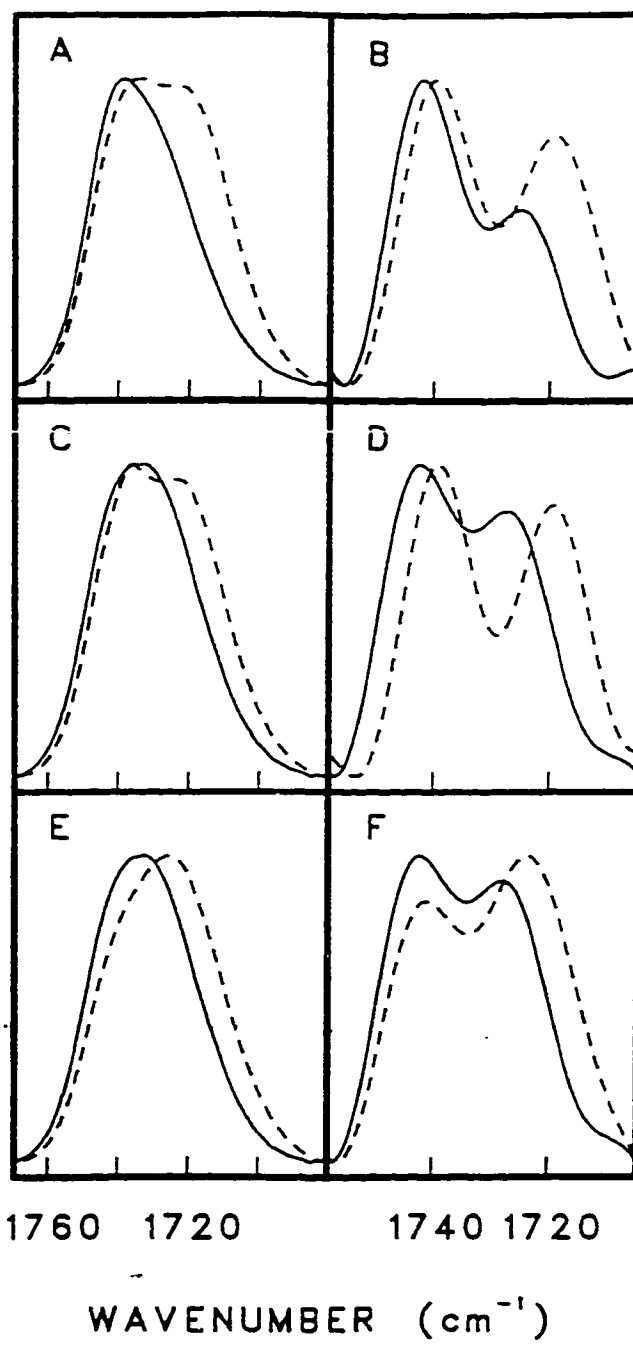


Figure 3.17 illustrates the absorption of the ester C=O bond of DMPC alone and the combined absorption of the ester C=O bond of SGG and the ester C=O bond of DMPC in mixed liposomes (2:3 molar ratio) in the gel phase (5°C) (Fig. 3.17A), at physiological temperature (37°C) (Fig. 3.17C) and in the liquid crystalline phase (55°C) (Fig. 3.17E). The Fourier deconvolved spectra of the same region are presented in Figures 3.17B, 3.17D and 3.17F. Upon deconvolution of the broad ester bands, at all temperatures, two component bands were revealed, one absorbing at low frequency and one absorbing at higher frequency. In all deconvolved spectra the higher frequency component bands were normalized to the same intensity.

In the gel phase, the broad ester band of DMPC is slightly wider than the absorption band of the ester bond in the mixed SGG+DMPC liposomes. However, there is no significant frequency shift. The high frequency components of the ester C=O bands of the mixed liposomes and DMPC liposomes both absorbed at 1743 cm^{-1} (Fig. 3.17B). Similarly, the lower frequency components of the mixed liposomes and DMPC liposomes both absorbed at 1725 cm^{-1} . The intensity ratio of the lower frequency component to the higher frequency component of the mixed liposomes decreased relative to the DMPC liposomes.

At physiological temperature (37°C), the ester band of the mixed liposomes appeared to have the same band shape and absorbing frequency as DMPC liposomes (Fig. 3.17C). The high frequency component of the ester C=O band of the mixed liposomes and DMPC liposomes both absorbed at 1744 cm^{-1} (Fig. 3.17D). The lower frequency component of both the mixed liposomes and the DMPC liposomes absorbed at 1727 cm^{-1} . Examination of the Fourier deconvolved spectra shows an increase in the relative intensity ratio of the low

frequency component to the higher frequency component of the mixed liposomes as compared to the mixed liposomes in the gel phase. However, this intensity ratio has still decreased as compared to DMPC liposomes.

Figure 3.17E shows that the ester C=O bond of the mixed liposomes and the DMPC liposomes absorbs at the same frequency with similar band shape. The high frequency component of the ester C=O band of the mixed liposomes absorbed at 1744 cm^{-1} and that of DMPC liposomes absorbed at 1745 cm^{-1} (Fig. 3.17F). The lower frequency components of the mixed liposomes and DMPC liposomes both absorbed at 1728 cm^{-1} . The relative absorbing intensities of both components in the mixed liposomes compared to the DMPC liposomes were almost identical.

The insertion of SGG into DMPC bilayers affected the absorbing intensity of the low frequency component in mixed liposomes, up to 37°C such that there were fewer ester C=O groups absorbing at low frequency in the mixed liposomes. This effect was no longer apparent at 55°C . The ester C=O frequency shifts of SGG, DMPC and the mixed SGG+DMPC liposomes are summarized in Table 4.

Figure 3.17 Infrared spectra of the ester C=O stretching region ($1760\text{--}1680\text{ cm}^{-1}$) of DMPC liposomes (— —) and SGG+DMPC (2:3 molar ratio) (——) at 5°C , in the gel phase (A); at 37°C , physiological temperature (C); and 55°C , in the liquid crystalline phase (E). (B), (D) and (F) are Fourier deconvolved spectra in (A), (C) and (E), respectively. Lipid samples were hydrated in 50 mM Tris-HCl in D_2O .

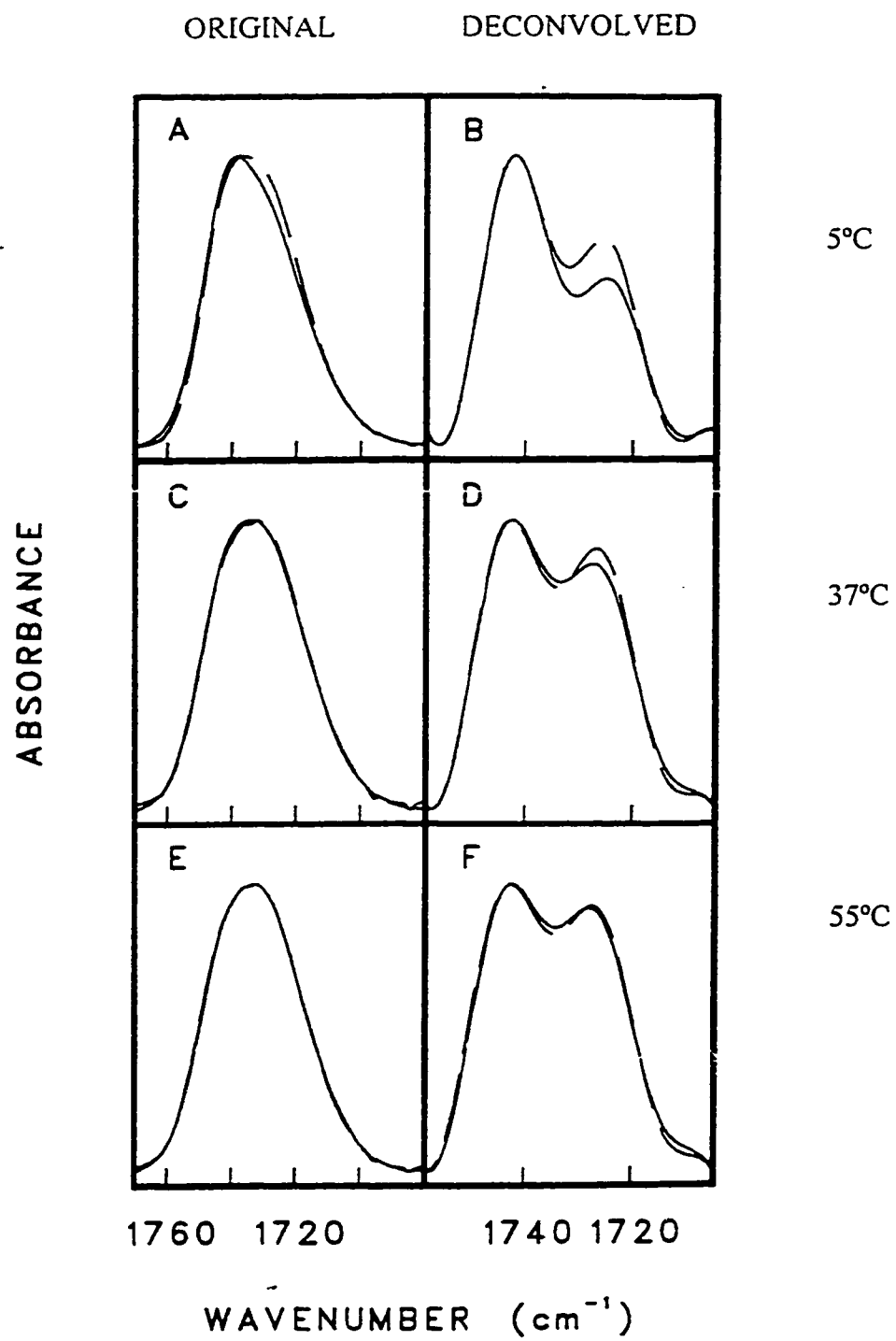


Table 4

Summary of the FTIR examination of ester C=O groups at the interfacial region of SGG, DMPC and SGG+DMPC liposomes^a.

Temperature (°C)	5		37		55	
Components (cm ⁻¹)	low	high	low	high	low	high
<u>Liposome System</u>						
SGG	1717	1741	1720	1737	1722	1743
DMPC	1725	1743	1727 1744		1728	1744
SGG+DMPC	1725	1743	1727 1744		1728	1744

^a See Figures 3.16 and 3.17 for the original deconvolved spectra.

3.2.6 Hydrocarbon Chain Region of SGG+DMPC

In order to investigate the effects of inserting DMPC into SGG bilayers, on the SGG hydrocarbon chains, absorption patterns in the CH_2 symmetric and antisymmetric stretching region were examined. Figure 3.18 presents the absorption of the CH_2 symmetric and antisymmetric stretching bands of SGG liposomes and SGG+deuterated DMPC liposomes (2:3 molar ratio) in the gel phase (5°C) (Fig. 3.18A), at physiological temperature (37°C) (Fig. 3.18B) and in the liquid crystalline phase (55°C) (Fig. 3.18C). The use of deuterated DMPC in the liposome mixture resulted in a shift of the absorption bands arising from deuterated DMPC hydrocarbon chains to a higher frequency, so that the observed CH_2 stretching bands represented those of SGG hydrocarbon chains. At all temperatures there was a shift of both the symmetric and antisymmetric CH_2 stretching absorption bands of SGG in the mixed liposomes to higher frequencies relative to SGG liposomes.

Figure 3.18A indicates that in the gel phase (5°C), the antisymmetric and symmetric CH_2 stretching bands of SGG in the mixed liposomes were shifted to 2920 cm^{-1} and 2852 cm^{-1} as compared to 2917 cm^{-1} and 2850 cm^{-1} , respectively, in the SGG liposomes. Furthermore there is an increase in bandwidth of these modes in mixed liposomes relative to the SGG liposomes.

Figure 3.18B shows that the antisymmetric and symmetric CH_2 stretching bands of SGG in the mixed liposomes are shifted to 2923 cm^{-1} and 2853 cm^{-1} relative to 2918 cm^{-1} and 2851 cm^{-1} in the SGG liposomes at physiological temperature (37°C). There is an increase in the bandwidth of these modes in the mixed liposomes relative to the SGG liposomes.

Figure 3.18C indicates that in the liquid crystalline phase (55°C), the antisymmetric

and symmetric CH_2 stretching bands of SGG in the mixed liposomes are shifted to 2924 cm^{-1} and 2854 cm^{-1} as compared to 2922 cm^{-1} and 2853 cm^{-1} in the SGG liposomes. The increase in bandwidth is no longer as apparent as was observed at lower temperatures.

At all temperatures there was a shift to higher frequency of both the symmetric and antisymmetric CH_2 stretching bands of SGG in mixed liposomes as compared to SGG liposomes. This indicated the insertion of deuterated DMPC into SGG bilayers resulted in an increase in conformational disorder of SGG hydrocarbon chains.

Figure 3.18 Infrared spectra of the CH_2 symmetric and antisymmetric stretching region ($3000\text{--}2800\text{ cm}^{-1}$) of SGG liposomes (---) and SGG+DMPC (2:3 molar ratio) (—) at 5°C , in the gel phase (A); at 37°C , physiological temperature (B); and at 55°C , in the gel phase (C). Lipid samples were hydrated in 50 mM Tris-HCl in D_2O .

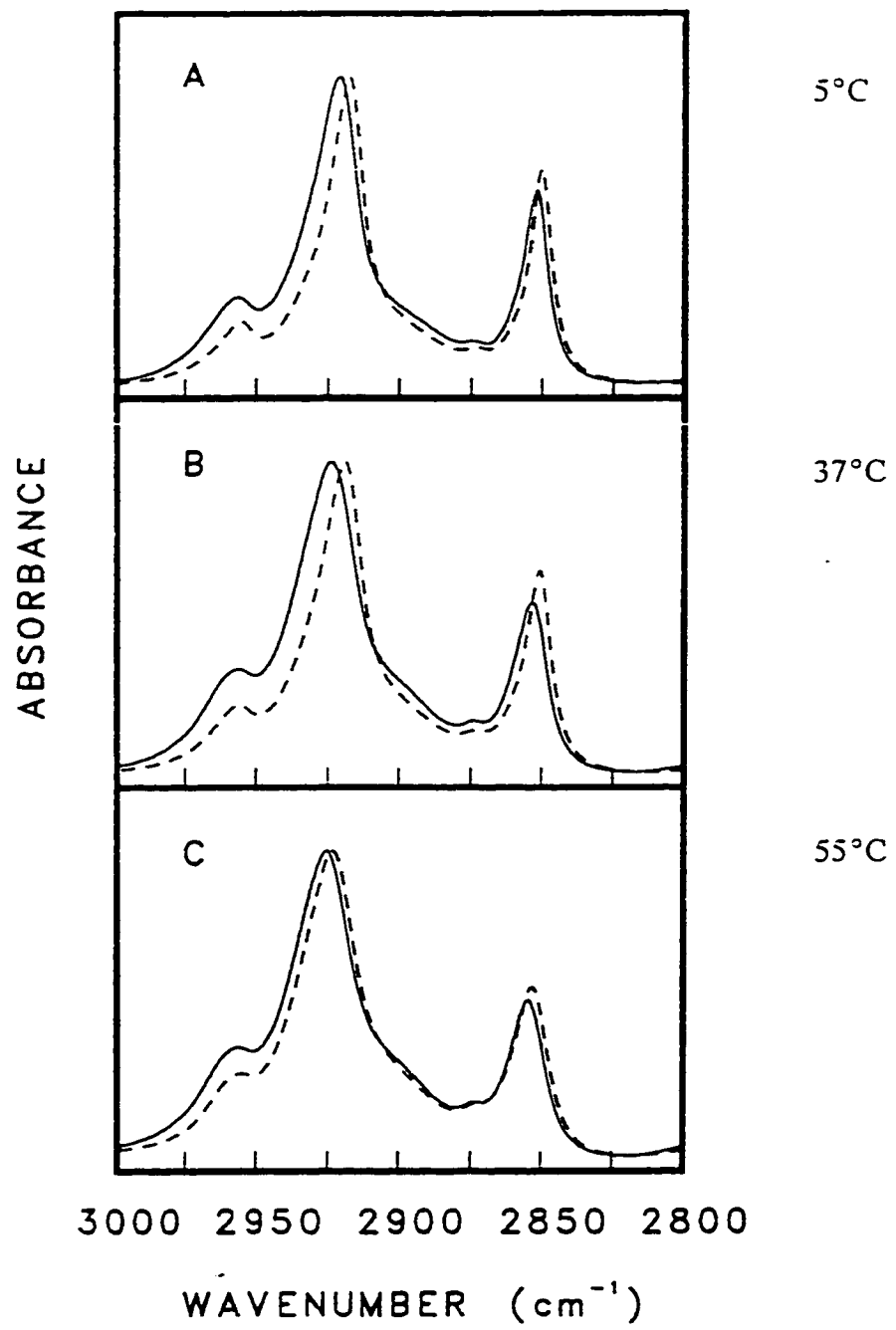


Table 5

Summary of the FTIR examination of the CH₂ symmetric and antisymmetric stretching vibrations of SGG and SGG+deuterated DMPC liposomes^a.

Temperature (°C)	5		37		55	
Components (cm ⁻¹)	sym.	anti.	sym.	anti.	sym.	anti.
<u>Liposome System</u>						
SGG	2850	2917	2851	2918	2853	2922
SGG+dDMPC ^b	2852	2920	2853	2923	2854	2924

^a See Figure 3.18 for the original spectra.

^b Mixed liposome system of SGG+deuterated DMPC (2:3, molar ratio).

CHAPTER FOUR

DISCUSSION

4.1 Biochemical Characterization of SGC and SGG

4.1.1 Thin Layer Chromatography Analysis of SGC and SGG

TLC was used to verify the purity of SGC and SGG samples. Both SGG and SGC were identified as pure glycolipids by positive staining with α -naphthol/ H_2SO_4 stain (Figs. 3.1 and 3.2). SGG was isolated from ram testes and found to have an R_f value of 0.28 which is in good agreement with the R_f value of 0.30 reported by Ishizuka et al. (1973).

4.1.2 Gas-Liquid Chromatography Analysis of SGC

The commercial SGC product used here was analyzed by GLC for non-hydroxy and α -hydroxy acids as described in Methods section. The major molecular species found to be present in non-hydroxy SGC subclass were C24:1 (51.5 %), C24:0 (14.5 %), C25:1 (9.8 %) and C26:1 (6.2 %) (weight % of the total non-hydroxy subclass) with lower proportions of C16:0, C18:0, C18:1, C22:0 and C23:0 (Table 1), in good agreement with the values reported by O'Brien and Rouser (1964). The major molecular species in the α -hydroxy SGC subclass were C24:1-h (49.7 %), C24:0-h (14.9 %) and C26:1-h (14.3 %) (weight % of the total hydroxy subclass) with lower amounts of C22:0-h, C23:0-h, C23:1-h and C25:1-h (Table 1). These results were also similar qualitatively to those reported by O'Brien and Rouser (1964) with some quantitative discrepancies, particularly with respect to the values for C24:0-h, C24:1-h and C18:0-h (Table 1). The weight ratio of hydroxy to non-hydroxy species was calculated to be 1:2.

With this information, we were aware that our mixed model system of SGC+DMPC (2:3, molar ratio) consisted of DMPC interacting with an asymmetric sphingoglycolipid with predominantly C24:1 as the acyl chain and one third of the SGC molecules were α -hydroxylated.

4.1.3 ^1H and ^{13}C NMR Analysis of SGG

One dimensional ^1H -NMR and two dimensional HMQC NMR were used for structure determination studies of SGG isolated from ram testes (Figs. 3.3 and 3.4). The chemical shifts summarized in Table 2 are in good agreement with ^1H -NMR results of Ishizuka et al. (1973) for boar testis SGG and Alvarez et al. (1990) for bovine testis SGG. The number of protons found through peak integration of the ^1H -NMR spectrum was 80.3 which is in good accord with the calculated number of protons based on the known structure of the free acid with C16:0 acyl and alkyl chains, 79. To the best of our knowledge, it was the first time that ^{13}C NMR (applied in 2D-HMQC) was used for SGG structural analysis. The carbon chemical shifts characterize the type of carbons with respect to protons and other functional groups that are bonded to the carbon atoms. Overall, our NMR results confirm the monoalkylmonoacyl-3'-sulfogalactosylglycerolipid (see Fig. 1.2) structure of the SGG isolated from ram testes for this study with respect to both proton and carbon atoms.

4.1.4 Fast Atom Bombardment-Mass Spectrometry Analysis of SGG

Negative FAB-MS of SGG was used to confirm the structure of SGG isolated from ram testes. The major ion peaks summarized in Table 3 are consistent with the known structure of SGG (see Fig. 1.2) and in good agreement with those reported by Ishizuka et al. (1973) and Alvarez et al. (1990). The ion peaks summarized in Appendix II (Table I)

correspond to minor molecular species of SGG that have been identified by GLC in our laboratory (Tupper et al., 1994).

4.1.5 Infrared Spectroscopic Characterization of SGG

The SGG purified from ram testes could be further characterized by examining its IR spectrum (Fig. 3.6). Isolated SGG had endogenous cations associated with the sulfate. These cations were removed and replaced with sodium ions using the acid Bligh and Dyer method (Kates, 1986). Free acid SGG molecules were neutralized with 0.05 N NaOH. The SGG ester group is alkaline labile, therefore, in order to verify the intactness of SGG, the interfacial region and hydrocarbon region were examined in the SGG IR spectrum. The presence of a CH_2 bending vibration (1467 cm^{-1}) and symmetric and antisymmetric CH_2 stretching vibrations ($\sim 2850\text{ cm}^{-1}$ and $\sim 2920\text{ cm}^{-1}$, respectively), as well as the carbonyl ester at $\sim 1735\text{ cm}^{-1}$ verified SGG was indeed intact.

4.1.6 Summary of Biochemical Characterization of SGC and SGG

The SGC used for this study was purchased from Sigma. SGC was isolated from bovine brain, where SGC is the major sulfoglycolipid of the myelin sheath (Murray et al., 1980; Murray and Narasimhan, 1990; Norton and Cammer, 1984) and occurs as α -hydroxy and non-hydroxy fatty acid containing species (O'Brien and Rouser, 1964; Koshy and Boggs, 1983). We verified the purity of commercial SGC with α -hydroxy and non-hydroxy subclasses by TLC and further characterized the molecular subclasses of the acyl chains through GLC analysis and determined the predominant species to be C24:1.

SGG was isolated and purified from ram testes, where it is the major sulfoglycolipid (Kornblatt et al., 1972; Ishizuka et al., 1973) using adsorption column chromatography.

TLC, NMR, FAB-MS and IR analysis all confirmed the structure of SGG and verified its purity.

4.2 FTIR Analysis of SGC and SGG

4.2.1 Thermotropic Analysis of SGC+DMPC

The physical state of bilayers will affect the rotational/vibrational order of membranes since the gel phase is by definition more ordered than the liquid crystalline state (Mantsch and McElhaney, 1991). Therefore, it was necessary to determine the physical state of SGC+DMPC (2:3 molar ratio) liposomes at the FTIR operating temperature of 28°C in order to be able to characterize the interaction between these two lipids. As lipid bilayers undergo the transition from the more ordered gel state to the more disordered liquid crystalline state the frequency of the CH₂ symmetric stretching vibration band increases from ~ 2850 cm⁻¹ to ~ 2854 cm⁻¹ due to an increase in the gauche/trans conformer ratio in the hydrocarbon chains (Mantsch and McElhaney, 1991). As well, the width of the CH₂ symmetric stretching band of the lipids will increase due to increased motional rates and to the larger number of conformational states of the hydrocarbon chains in the liquid crystalline state (Mantsch and McElhaney, 1991). The fact that SGC contains many molecular species, as revealed by our GLC analysis of SGC, had to be taken into account when examining the interaction between the two molecules. This problem did not exist in the analysis of SGG+DMPC liposomes since SGG contains fewer molecular subclasses, C16:0 is the predominant acyl and alkyl chain subclass as revealed by previous GLC analysis in our laboratory (Tupper et al., 1994).

Using the CH_2 symmetric stretching band of methylene chains, we found the gel-to-liquid crystalline phase T_m to be 42°C for the mixed liposomes containing SGC+DMPC. As expected, this T_m of the mixed liposomes was between those of the individual lipids, i.e., 23.6°C for DMPC (Bou Khalil and Carrier, personal communication) and 50.3°C for synthetic C24:1 SGC (Boggs et al., 1988) or $61\text{--}80^\circ\text{C}$ for other components of the SGC commercial sample (Boggs et al., 1993). Since the T_m is well above the FTIR operating temperature, the mixed liposomes were in the gel phase when ambient pressure FTIR measurements were taken. The presence of a single and cooperative transition in Fig. 3.7 is consistent with a homogeneous mixing of SGC and DMPC at a molar ratio of 2:3 within the bilayers without lateral phase separation.

4.2.2 Hydrogen Bonding at the Interfacial Region of SGC+DMPC liposomes

The C=O stretching vibrations from the interfacial regions of SGC and DMPC are sensitive to both conformational and environmental factors (Mantsch and McElhaney, 1991). The degree of interfacial hydrogen bonding can be assessed by examining the amide I stretching mode (ranging from $1630\text{--}1670\text{ cm}^{-1}$) and the ester C=O stretching mode (ranging from $1700\text{--}1750\text{ cm}^{-1}$) (Kates, 1986). The shifts in these stretching frequencies in the mixed liposomes, as compared to that in the single lipid liposomes, can be used to monitor changes in interfacial hydrogen bonding. Figures 3.8A and 3.9A illustrate that both the amide I band of SGC and the ester C=O band of DMPC shifted to higher frequencies in the mixed liposomes of SGC+DMPC, as compared to the corresponding values for the SGC liposomes and DMPC liposomes. The amide I band arising from SGC in the mixed liposomes shifted to a frequency of 1645 cm^{-1} compared to a frequency of 1624 cm^{-1} for the SGC liposomes

(Fig. 3.8A). The band arising from the ester C=O of DMPC in the mixture with SGC was shifted slightly to a higher frequency (1741 cm^{-1}) as compared to the DMPC liposomes (1738 cm^{-1}) (Fig. 3.9A). This indicates that both the amide C=O and the ester C=O in the mixture had an increased double-bond character, which likely reflected mutual shielding of SGC and DMPC by one another and a consequent reduction of hydrogen bonding.

Deconvolution of either C=O band revealed a low frequency component and a high frequency component in both the single lipid liposomes and the mixed liposomes (Figs. 3.8B and 3.9B). The frequency of the C=O stretching band decreases with increased involvement in hydrogen bonding (Wong and Mantsch, 1988). However, interpretation of the identity of each of the deconvolved component bands that contribute to the broadness of either the original amide C=O band of SGC or ester C=O band of DMPC is not straightforward.

Tupper et al. (1992) have assigned the high frequency component at $\sim 1640\text{ cm}^{-1}$ in the deconvolved spectrum of the amide C=O band of SGC to the non-hydroxy SGC subclass, which is less involved in hydrogen bonding, and the low frequency component at 1619 cm^{-1} to the α -hydroxy SGC subclass which is involved in intramolecular hydrogen bonding of the NH moiety of the amide group with the α -hydroxy group. This hydrogen bonding would cause an increase in electron density at the amide C=O and hence an increase in its dipole moment, which would promote stronger hydrogen bonding of the amide C=O with either the sphingosine OH group or the galactose OH groups of the neighboring SGC molecules (Tupper et al., 1992; Nabet et al., 1996). This interpretation was based on FTIR studies performed with pure hydroxy and non-hydroxy galactosylceramide (GC) species each of which gave a single amide band (Tupper et al., 1992). In the present study, the low

frequency component of the amide I group of SGC was shifted from a frequency of $\sim 1619\text{ cm}^{-1}$ in the SGC liposomes to $\sim 1626\text{ cm}^{-1}$ in the mixed liposomes. The higher frequency component (1640 cm^{-1}) of SGC shifted to a frequency 1648 cm^{-1} in the mixed liposomes (Fig. 3.8B). These shifts to higher frequencies of both components reflect a decrease in the degree of hydrogen bonding of the amide C=O in SGC (Wong and Mantsch, 1988). Furthermore, the intensity of the higher frequency component increased concomitantly with a decrease in the intensity of the low frequency component. Thus, the proportion of weakly hydrogen bonded SGC molecules appears to have increased along with a decrease in the distribution of more strongly hydrogen bonded SGC molecules. This effect may be explained by the insertion of DMPC molecules between SGC molecules in the mixed membranes, which would shield SGC molecules from hydrogen bonding with neighboring SGC molecules.

However, a recent study has shown that more than one component band was observed upon deconvolution of the amide C=O band of either pure non-hydroxy SGC or hydroxy SGC (Menikh et al., 1997). Apparently, this result challenges the validity of the earlier interpretation by Tupper et al. (1992) that the two individual component bands could be assigned unambiguously to non-hydroxy (high frequency band) or hydroxy SGC (low frequency band) subclasses. To clarify this situation, we have obtained the spectrum of pure non-hydroxy SGC (C24:1) (data not shown) in the sodium salt form which we used throughout this study as compared to the ammonium salt form used by Menikh et. al (1997). The amide I band of the C24:1 non-hydroxy SGC species was centered at $\sim 1648\text{ cm}^{-1}$ and could be deconvolved into two component bands at 1631 cm^{-1} and 1651 cm^{-1} . The lower

frequency band (1631 cm^{-1}) may represent hydrogen bonded non-hydroxy SGC species and the higher frequency band (1651 cm^{-1}) may represent non-hydrogen bonded non-hydroxy SGC species. Therefore, the low frequency band at 1619 cm^{-1} obtained upon deconvolution of the broad amide I band of bovine brain SGC (sodium salt form containing both hydroxy and non-hydroxy SGC subclasses) (Fig. 3.8B) likely arises from the absorbance of the hydrogen bonded hydroxy SGC species and the high frequency band at 1648 cm^{-1} likely arises from absorbance of the non-hydroxy SGC species.

Figure 3.9B displays the deconvolved spectra of the ester C=O stretching region of DMPC. The broad band arising from the ester C=O of DMPC in the mixture with SGC was shifted slightly from 1738 cm^{-1} in the DMPC liposomes to a higher frequency 1741 cm^{-1} . Fourier deconvolution of this ester C=O band of DMPC reveals low frequency and high frequency components (Fig. 3.9B). When the higher frequency components of the DMPC liposomes and the DMPC + SGC liposomes were normalized to the same intensity, the intensity ratio of the lower frequency component relative to the higher frequency component was decreased in the mixed liposomes, as compared to the ratio observed for the DMPC liposomes, without any apparent frequency shift of the lower frequency band (Fig. 3.9B). These results may be explained by a decrease in the transition moment of ester C=O groups absorbing at the low frequency in the mixed liposomes, due to changes in the environment caused by the presence of SGC. The latter changes may also be involved in the shift of the higher frequency component observed in the mixed liposomes. It is plausible that the introduction of SGC, with its electronegative groups at the interfacial region, into the bilayers would decrease the transition moment of the ester C=O group, thus reducing its absorption

frequency. Therefore the slight band shift of the high frequency band and change in intensity of the low frequency band may result from a general reduction in hydrogen bonding of the ester C=O groups.

The broadness of the original spectrum of the ester C=O group has been reported to be attributable to both *sn*-1 chain and *sn*-2 chain absorptions, which are not equivalent (Yeagle and Martin, 1976; Wong and Mantsch, 1988). The Fourier deconvolved spectra reveal low and high frequency components, reflecting contributions from the *sn*-2 and *sn*-1 C=O groups, respectively. Previous work has indicated that the *sn*-2 ester C=O group being more exposed to the aqueous medium, participates to a greater extent in hydrogen bonding, as compared to the *sn*-1 C=O, which is more shielded (Wong and Mantsch, 1988). This interpretation has been questioned on the basis of experiments in which the *sn*-1 or the *sn*-2 chain C=O carbon atom was selectively substituted with ^{13}C isotope (Blume et al., 1988; Lewis et al., 1994). The latter studies suggested that both the *sn*-1 and *sn*-2 carbonyl groups can contribute to the high frequency and low frequency absorption bands, and that the high frequency may have arisen from free and mono hydrogen bonded ester C=O groups, whereas the low frequency component arose from di, tri and higher orders of hydrogen bonded ester groups. However, these conflicting arguments do not affect our conclusion that the interaction of DMPC and SGC in the mixed liposomes resulted in an overall reduction of hydrogen bonding of the ester C=O groups of DMPC.

4.2.3 Hydrocarbon Chain Region of SGC+DMPC

Pressure-tuning FTIR spectroscopy has been used to evaluate the dynamic properties of lipid bilayers. The methylene bending mode absorption band (δCH_2) (1450 to 1490 cm^{-1})

is usually used for this purpose, since it is sensitive to structural differences in hydrocarbon chain packing (Stein and Sutherland, 1953, 1954). Increasing pressure induces correlation field splitting of this bending frequency, and the pressure at which this splitting is initially observed being directly related to membrane fluidity (Wong et al., 1988; Wong, 1994).

Figure 3.10 presents the stacked contour plots of the δCH_2 bending mode of the SGC+deuterated DMPC bilayers. The results of our previous studies on SGC liposomes showed a correlation field splitting of the δCH_2 absorption band at 5.1 kbar (Tupper et al., 1992). In contrast, in the mixed liposomes, a broadening of the δCH_2 absorption band with no visible correlation field splitting was observed in the spectra with increasing pressure up to 13.2 kbar (Fig. 3.10). However, calculation of maximal δCH_2 bending frequencies with increasing pressure from the third order derivative spectra revealed that the onset of correlation field splitting was experimentally detected at 7.7 kbar and two frequency components were first detected at 9.2 kbar (Fig. 3.11). This may be explained by postulating that both of the SGC hydrocarbon chains were still undergoing reorientational fluctuations in the pressure range up to 13.2 kbar resulting from the high degree of orientational and conformational disorder introduced by the presence of DMPC in the mixed liposomes. These fluctuations would lead to a broadening of the correlation field component bands which would overlap into one broad band rather than separating into two bands. The disorder introduced by DMPC in the mixed liposome bilayers, may in part be attributable to the discrepancy in chain length between the major fatty acyl chains of both non-hydroxy and α -hydroxy SGC (C24:1 and C24:1-h, respectively) and the DMPC fatty acyl chains (C14:0) which could potentially lead to void areas in the bilayers. However, the lipids would tend

to undergo reorientational and conformational movements to eliminate such hypothetical void areas at all pressures.

Figure 3.12 presents the stacked contour plot of the methylene bending mode region of the mixed liposomes of SGC+DMPC (not deuterated). The spectra reflect contributions from both DMPC hydrocarbon chains and SGC hydrocarbon chains. Figure 3.13, displaying the pressure dependence plot of the δCH_2 bending mode frequencies, reveals an experimentally detected onset of splitting at ~ 4 kbar, with the initial appearance of an observable shoulder at the high frequency side of the δCH_2 bending vibration at 5.3 kbar. The intensity of the high frequency shoulder/peak rose steadily with increasing pressure until a well defined component was apparent at 7.7 kbar. The observation of only a single CH_2 bending absorption band at atmospheric pressure reflected the fact that under these conditions of temperature and pressure, all of the methylene chains were highly disordered in the mixed liposomes due to mutual interaction of SGC and DMPC molecules, as compared to the hydrocarbon chains in the individual lipid liposomes. Increasing pressure would lead to dampening of the reorientational fluctuations and an increase in interchain interactions, giving rise to the vibrational coupling responsible for correlation field splitting (Auger et al., 1988). Studies on liposomes of DMPC or SGC have shown that the correlation field splitting occurs at 3.2 kbar (Auger et al., 1988; Wong et al., 1988; Wong, 1994) and 5.1 kbar (Tupper et al., 1992), respectively. It is therefore likely that the onset of splitting observed at ~ 4 kbar in the mixture of SGC and DMPC (Fig. 3.13) was due to the splitting of the vibrational modes of the DMPC hydrocarbon chains. The higher pressure required to observe the splitting in the mixed liposomes (~ 4 kbar), as compared to that of the DMPC

liposomes (3.2 kbar), indicated that the presence of SGC resulted in a greater degree of orientational disorder of DMPC hydrocarbon chains. However, a second splitting attributable to the SGC δCH_2 bending mode band was not observed in the pressure dependence plot of the SGC+non-deuterated DMPC liposomes (Fig. 3.13). This is likely due to the possibility that SGC's signal at that frequency was weaker than that of DMPC and probably overlapped with that of DMPC. Furthermore, comparison of the stacked contour plot of the SGC+non-deuterated DMPC liposomes (Fig. 3.12) with that of the pure DMPC liposomes (Auger et al., 1988; Wong et al., 1988; Wong, 1994) showed differences not only in the splitting pressure (3.2 kbar for DMPC versus ~ 4 kbar for SGC+non-deuterated DMPC) but also in the shape of the spectra, which reflects the heterogeneity of the mixed molecular system, with respect to acyl chain types. The barotropic results also support the thermotropic observations (Fig. 3.7), both suggesting the virtual absence of lateral phase separation. The fact that the vibrational splitting pressures observed for each lipid present in the mixed liposomes were different from those of the corresponding single lipid liposomes suggests that the two lipid components when mixed, formed a homogeneous mixture with cross-interaction of the acyl chain and the polar head groups between the two types of lipids. Furthermore, the lack of a "valleying" pattern in the stacked contour plots (Figs. 3.10 and 3.12) suggests the lipids were not packed such that the hydrocarbon chains were interdigitated.

4.2.4 Summary of FTIR Analysis of SGC+DMPC liposomes

The present results show that SGC and DMPC in the mixed liposomes (molar ratio 2:3) form a homogeneous mixture, consistent with the absence of lateral phase separation.

The HP-FTIR study of the methylene bending mode of the mixed liposomes (SGC+DMPC) revealed that the co-presence of the two lipids resulted in an increase of hydrocarbon chain orientational and conformational disorder (fluidity) of both lipid components. This observed increase in fluidity is consistent with a decrease in interfacial hydrogen bonding in the mixed liposomes, as compared to that in the individual lipid liposomes. Disruption in hydrogen bonding at the interfacial region would allow for more rotational freedom of the hydrocarbon chains thereby increasing their observed orientational disorder.

This model study of SGC+DMPC liposomes, employing thermotropic and barotropic FTIR, as well as FTIR at ambient pressure and temperature, provides a model approach for further investigation of membrane lipids. However, the use of HP-FTIR for the study of SGC+DMPC liposomes poses a problem since the correlation field splitting values of SGC (3.3 kbar) (Tupper et al., 1994) and DMPC (3.2 kbar) (Auger et al., 1988; Wong et al., 1988; Wong, 1994) are so close that it would be difficult to determine the identity of which of the two lipids was responsible for any observed correlation field splitting in mixed liposomes. However, thermotropic FTIR could be used in a similar manner, as used in this study for T_m determination. Furthermore, one can expand the use of thermotropic FTIR to analyze different structural groups at different temperatures which would give information as to the interaction of membrane lipids in different physical states (i.e., gel state versus liquid crystalline state).

4.2.5 Thermotropic Analysis of SGC and SGC+DMPC liposomes

While the T_m of DMPC is known to be between 23-24°C (Wong and Mantsch, 1985; Bou Khalil and Carrier, personal communication), SGC's T_m has never been determined.

Previous differential scanning calorimetry studies of intact sperm membranes indicated two T_m 's of $\sim 26^\circ\text{C}$ and $\sim 60^\circ\text{C}$ (Wolf et al., 1990). The first T_m was interpreted to be from the phase transition of phospholipids, the major structural lipids of the sperm plasma membrane, while the second T_m was interpreted to be from the phase transition of germ-cell specific, SGG. This interpretation also implied that SGG is segregated from other lipids in the sperm plasma membrane. The purpose of this study was to examine the interaction between SGG and DMPC in lipid bilayers.

Using the CH_2 symmetric stretching vibration, the gel-to-liquid phase T_m of SGG was found to be 45°C and of SGG+DMPC (2:3 molar ratio) to be 28°C . The T_m of DMPC has been determined to be 23.6°C , in the laboratory of Dr. Danielle Carrier, at the University of Ottawa, in which we carried out our thermotropic analyses. The T_m of the mixed liposomes was observed to be between the phase transition temperatures of SGG and DMPC. However, the T_m of the mixed liposomes was closer to the T_m of DMPC which indicated that DMPC had a greater effect as a "fluidizer" acting on SGG than SGG's effect acting as a "rigidifier" on DMPC. DMPC being present in a greater amount in the liposomes would be expected to have a greater effect on SGG. However, in comparison to the similar situation in the model system of SGC+DMPC (2:3 molar ratio) we observed that DMPC did not have as great an effect on SGC as a "fluidizer" or conversely SGC may have had a greater effect as a "rigidifier" on DMPC when compared to SGG. The SGG+DMPC mixed liposomes have a lower transition temperature (28°C) than the SGC+DMPC mixed liposomes (42°C). Therefore, less energy is required to produce a transition from the gel phase to the liquid crystalline phase indicating that the SGG+DMPC system is a more disordered system than

the SGC+DMPC system. Ultimately, the difference in the transition temperatures between the two systems can be attributed to the difference in the molecular structure of SGG versus SGC. SGC's interfacial region has the potential for more intramolecular and intermolecular hydrogen bonding due to the presence of the α -hydroxy group in one third of the SGC molecules (i.e., the ratio of hydroxy to non-hydroxy subclass was found to be 1:2 in this commercial SGC sample (section 3.1.2)) which could lead to a more ordered system through stabilization of the gel phase through a hydrogen bonding network. Furthermore, Boggs et al. (1981) have reported lamellar phase destabilization by alk-1'-enyl or alkyl chains in position *sn*-1 of ethanolamineglycerolphospholipids. SGG has an alkyl group in ether linkage at position *sn*-1 and thus may have a similar effect in destabilizing lamellar phases as the reported ethanolamineglycerolphospholipids.

The position of the symmetric C-H stretching vibration is a measure of the number of gauche conformers in the acyl chains. When all the methylene groups are in trans conformation, the band is observed at $\sim 2849\text{ cm}^{-1}$. Addition of gauche conformers results in a shift to higher frequencies (Umemura et al., 1980; Mantsch and McElhaney, 1991; Reis et al., 1996). At 5°C , the first temperature at which CH_2 symmetric stretching was monitored, DMPC liposomes, SGG liposomes, and SGG+DMPC liposomes were all in the gel phase (Fig. 3.14). It was observed that the CH_2 absorption frequency of DMPC (2849.6 cm^{-1}) was the lowest followed by SGG liposomes (2850.2 cm^{-1}) and finally SGG+DMPC mixed liposomes (2850.5 cm^{-1}), which absorbed at the highest frequency. As expected, the single lipid liposomes were more ordered in the gel phase and contained less gauche conformers than the mixed lipid liposomes as indicated by the frequency of their symmetric

CH₂ stretching vibration. Thus, the interaction of SGG and DMPC in bilayers led to an increase in gauche conformers and thus to a less tightly packed, more disordered system.

All three temperature profiles showed one smooth main transition. This is significant in the mixed liposomes, since one main transition is indicative of homogeneous mixing of SGG and DMPC, with an absence of lateral phase separation. Differential scanning calorimetry studies on ram sperm plasma membranes have suggested SGG is phase segregated in the plasma membrane and may be responsible for an endothermic transition observed at ~ 60°C (Wolf et al., 1990). Our results do not confirm this observation. Our lipids were indeed interacting in the mixed liposome system and furthermore, the T_m of SGG was determined to be 45°C which is significantly lower than the observed transition at ~ 60°C in ram sperm. However, the system we studied was only a model system that lacked many important parameters that would also affect membrane dynamics in a natural system (i.e., other lipids and proteins) (Shinitzky, 1984). In particular, cholesterol will affect membrane dynamics and its high content in human sperm plasma membranes was suggested to affect thermotropic behaviour such that it was difficult to detect a thermotropic phase transition in human sperm membranes (Drobnis et al., 1993). It is plausible that in a natural system cholesterol acting as a “rigidifier” to the membrane, may result in a phase separation of glycolipids as well as an increase in their transition temperatures. To further elucidate this question as to the phase behaviour of SGG in sperm plasma membranes, more complex model systems must be studied.

The temperature profile of SGG liposomes indicated SGG also underwent a pretransition before the main gel-to-liquid crystalline phase transition. The onset of the

pretransition was at 20°C and was complete upon the onset of the main transition at 41°C. This pretransition could also be monitored by observing the changes to the ester C=O band (Fig. 3.15). Between 20-41°C, there appeared two distinct bands, a low frequency component (1719 cm⁻¹) and a high frequency component (1739 cm⁻¹). At 20°C, the high frequency shoulder of the C=O band started to increase in intensity and both components began to become narrower. Mantsch et al. (1982) studied the polymorphic phase behaviour of phosphatidylsulfocholine bilayers using FTIR spectroscopy and demonstrated that general band narrowing and an increase in peak height of methylene chain absorptions is indicative of a reduction in the mobility of the acyl chains. Furthermore, rotation about the C-2-C-1 bond of the glycerol backbone is possible (Wong and Mantsch, 1988) and the ester C=O bond absorption is sensitive to the geometry of the glycerol moiety and the packing of the acyl chains (Mantsch and McElhaney, 1991). One can thus speculate that the increase in intensity of the high frequency component and the band narrowing of both components of the ester C=O band may reflect a transition of the SGG molecules in the bilayers into a less mobile arrangement. This phase behaviour would be analagous to the reported pretransition of GC bilayers from a metastable gel phase polymorph to a stable gel phase polymorph (Curatolo et al., 1982, Curatolo, 1985). The disappearance of the two component bands of the ester C=O groups upon onset of the main gel-to-liquid phase transition would be a result of increased mobility of the lipid molecules as they undergo transition to the more fluid (disordered) liquid crystalline state.

A pretransition was not observed in the mixed SGG+DMPC liposomes. This indicated that the insertion of DMPC into SGG bilayers may have possibly resulted in the

loss of more ordered gel phase polymorph that may exist in SGG bilayers.

4.2.6 Hydrogen Bonding at the Interfacial Region of SGG+DMPC liposomes

The interactions between SGG and DMPC at the interfacial region were studied by examining the ester C=O stretching band region ($1700\text{--}1750\text{ cm}^{-1}$) (Kates, 1986). This region is sensitive to both conformational and environmental factors (Mantsch and McElhaney, 1991).

Figure 3.16 illustrates the absorption of the ester C=O bond of SGG liposomes and the combined absorption of the ester C=O bond of SGG and the ester C=O bond of DMPC in mixed liposomes (2:3 molar ratio) in the gel phase (5°C), at physiological temperature (37°C) and in the liquid crystalline phase (55°C). At all temperatures, the ester C=O groups in the mixed liposome system (SGG+DMPC, 2:3 molar ratio) absorbed at a higher frequency than the ester C=O groups of SGG liposomes. This indicates the ester C=O groups of the mixed liposome system had an increased double-bond character, which likely reflected mutual shielding resulting from interaction between SGG and DMPC and a consequent reduction of hydrogen bonding of each lipid in the mixture. Upon deconvolution of the broad ester bands, two component bands were revealed at all temperatures, one absorbing at low frequency and one absorbing at higher frequency. At all temperatures, the high frequency component of the mixture did not significantly shift. These ester C=O groups absorbing at higher frequency were likely weakly hydrogen bonded or non-hydrogen bonded (Blume et al., 1988; Lewis et al., 1994) and thus were not greatly affected by increasing temperature.

In the gel phase (5°C), the broad ester C=O band of SGG liposomes was wider than

that of the mixed SGG+DMPC liposomes. Therefore, it appeared there was a group of strongly intermolecularly hydrogen bonded SGG molecules in SGG liposomes that were absent in the mixture (Fig. 3.16A). Examining these spectra after deconvolution revealed that indeed there was group of SGG molecules absorbing at 1717 cm^{-1} in SGG liposomes, while the low frequency component of mixed liposomes was located at 1725 cm^{-1} . It seems likely that the insertion of DMPC molecules into SGG bilayers disrupted intermolecular hydrogen bonding of the ester C=O groups that absorbed at 1717 cm^{-1} in the SGG liposomes. There was a slight band shift of 2 cm^{-1} to higher frequency of the higher frequency component of mixed liposomes relative to SGG liposomes. When the higher frequency components of the SGG liposomes and the SGG+DMPC liposomes were normalized to the same intensity, the intensity ratio of the lower frequency component relative to the higher frequency component was decreased in the mixed liposomes, as compared to the ratio observed for the SGG liposomes (Fig. 3.16B). These results may be explained by a decrease in the number of more strongly hydrogen bonded ester C=O groups upon insertion of DMPC into SGG bilayers. It is plausible that the introduction of DMPC into the SGG bilayers shielded SGG molecules from their intermolecular hydrogen bonding partners. Therefore, the slight band shifts to higher frequencies of both components in the deconvolved spectra as well as the change in intensity of the low frequency component band may have resulted from a general reduction in hydrogen bonding of the ester C=O groups in the mixed SGG+DMPC liposome system.

At physiological temperature (37°C), there was a down shift of the high frequency component of SGG liposomes (1737 cm^{-1}) as compared to SGG liposomes in the gel phase

(1741 cm^{-1}) (Fig. 3.16D). This indicated that these ester C=O groups absorbing at higher frequency were more involved in hydrogen bonding at physiological temperature as compared to the situation at 5°C. This increased involvement in hydrogen bonding of ester C=O groups of SGG liposomes would support the possible existence of a metastable gel phase polymorph at low temperatures and a stable gel phase polymorph at higher temperatures but still below the T_m (T_m of SGG=45°C). Furthermore, comparison of SGG liposomes versus SGG+DMPC liposomes again revealed that both the high frequency component and low frequency component of the ester C=O groups of mixed liposomes were shifted to higher frequencies. This indicated that the insertion of DMPC in mixed bilayers with SGG resulted in a disruption of SGG intermolecular hydrogen bonding. The intensity ratio of the lower frequency component to the higher frequency component of mixed liposomes increased at physiological temperature as compared to the gel phase. An increase in this ratio is indicative of an increase in the number of more strongly hydrogen bonded ester C=O groups at 37°C versus 5°C, possibly reflecting the increased mobility of the mixed liposomes that were in the liquid crystalline phase at 37°C. Increased mobility of the hydrocarbon chains would likely lead to a less tightly packed hydrophobic region. Under these circumstances water molecules could penetrate into the bilayers with more ease and hydrogen bond at the interfacial region of the lipid molecules.

At 55°C, both the high frequency component and low frequency component of ester C=O groups in the mixed liposomes absorbed at a higher frequency than the SGG liposomes. This indicated that even at high temperatures when both liposome systems were in the liquid crystalline phase hydrogen bonding of the mixed liposomes was disrupted as compared to

SGG liposomes. Interestingly, there was an increase in the intensity of the low frequency component of SGG liposomes with concomitant decrease in the high frequency component. Thus, the proportion of weakly hydrogen bonded SGG molecules decreased with an increase in more strongly hydrogen bonded molecules upon transition into the liquid crystalline phase. This may be a result of increased mobility of SGG hydrocarbon chains allowing more water to penetrate and more strongly hydrogen bond with the ester C=O group of SGG molecules.

Overall, the insertion of DMPC into SGG bilayers resulted in disruption of intermolecular hydrogen bonding of SGG at all temperatures. This may be a result of a shielding effect that DMPC had on SGG, such that SGG was no longer able to intermolecular hydrogen bond with other SGG molecules. The reduction of intermolecular hydrogen bonding between SGG molecules would result in a more mobile and disordered system. Furthermore, it would be plausible that a more stable gel phase polymorph with reduced acyl chain mobility that may exist in SGG bilayers would no longer exist in this more disordered system.

Figure 3.17 illustrates the absorption of the ester C=O bond of DMPC liposomes and the combined absorption of ester C=O bond of SGG and ester C=O bond of DMPC in mixed liposomes (2:3 molar ratio). At all temperatures examined in this study there were no significant frequency shifts upon insertion of SGG into DMPC bilayers of the broad ester bands (Figs. 3.17A, C, E) or the low and high frequency component bands revealed upon deconvolution (Figs. 3.17B, D, F). However, changes in the intensity of the lower frequency band of deconvolved spectra were observed whose higher frequency components were normalized to the same intensity.

In the gel phase (5°C), the intensity ratio of the lower frequency component relative to the higher frequency component was decreased in the mixed liposomes, as compared to the ratio observed for the DMPC liposomes (Fig. 3.17B). This may result from a decrease in the transition moment of ester C=O groups absorbing at the lower frequency in the mixed liposomes, due to changes in the environment caused by the presence of SGG. It is plausible that the introduction of SGG, with its electronegative groups at the interfacial region, into the bilayers would decrease the transition moment of the ester C=O group of DMPC, thus reducing its absorption frequency. Furthermore, a reduction in transition moment would reduce the hydrogen bonding potential of these ester C=O groups, thus leading to a disruption of interfacial hydrogen bonding.

At physiological temperature (37°C), a decrease in the relative intensity ratio of the low frequency component to the high frequency component of mixed liposomes relative to DMPC liposomes was still observed but to a lesser extent. Nevertheless, at physiological temperature the spectra still indicated that insertion of SGG into DMPC bilayers reduced the transition moment of the ester C=O groups absorbing at low frequency. Thus, the hydrogen bonding potential of these groups was also expected to decrease.

At 55°C, ester C=O groups of DMPC liposomes and ester C=O groups of SGG+DMPC liposomes had virtually identical absorption patterns. Both liposome systems were well into the liquid crystalline phase and were thus expected to be highly mobile and disordered.

The insertion of SGG into DMPC bilayers resulted in a decrease in the transition moment of DMPC ester C=O groups up to 37°C. Thus, this decrease in transition moment

was likely accompanied by a disruption of the hydrogen bonding network at the interfacial region. At 55°C, the effect of SGG insertion into DMPC bilayers relative to the absorption pattern of DMPC ester groups was no longer evident.

4.2.7 Hydrocarbon Chain Region of SGG+DMPC

The CH₂ symmetric and antisymmetric stretching vibrations are characteristic of the hydrophobic hydrocarbon region (Mantsch and McElhaney, 1991). The frequencies of these bands are conformation sensitive and will shift in response to changes in the trans/gauche ratio of the acyl chains (Mantsch and McElhaney, 1991). For the SGG+DMPC study we chose thermotropic FTIR to investigate the effect of interaction of SGG and DMPC on the hydrocarbon region. HP-FTIR was not used to examine the hydrocarbon region, as was done for the SGC+DMPC investigation since the correlation field splitting pressures of SGG (3.3 kbar) (Tupper et al., 1994) and DMPC (3.2 kbar) (Auger et al, 1988; Wong et al., 1988; Wong, 1994) were so close that it would be difficult to determine which lipid was responsible for correlation field splitting observed in a mixed model system. Furthermore, time constraints and inaccessibility to HP-FTIR equipment also made the study impossible to plan and carry out.

Figure 3.18 presents the CH₂ symmetric and antisymmetric stretching bands of SGG liposomes and SGG+deuterated DMPC liposomes (2:3 molar ratio) in the gel phase (5°C) (Fig. 3.18A), at physiological temperature (37°C) (Fig. 3.18B) and in the liquid crystalline phase (55°C) (Fig. 3.18C). The use of deuterated DMPC allowed for selective examination of SGG hydrocarbon chains in the mixture. At all temperatures, both the symmetric and antisymmetric CH₂ stretching bands were shifted to higher frequency in the mixed liposome

system. This indicated that insertion of DMPC into SGG bilayers resulted in an increase in the number of gauche conformers in SGG hydrocarbon chains. Introduction of gauche conformers would lead to less tightly packed hydrocarbon chains and thus a more disordered hydrocarbon region. This was supported by the increase in bandwidth of SGG symmetric and antisymmetric CH₂ stretching vibrations which was especially apparent at 37°C. This increase in bandwidth reflected increased motional rates and an increased number of conformational states of the hydrocarbon chains which was consistent with a more disordered system.

4.3 Significance of Research Findings

In summary, our present results revealed that both SGC and SGG formed a homogeneous mixture with DMPC in the molar ratio 2:3 (sulfoglycolipid:phospholipid) with an absence of lateral phase separation. For the first time the T_m of SGG was determined to be 45°C. Comparison of the T_m of SGC+DMPC liposomes (42°C) versus SGG+DMPC liposomes (28°C) revealed that the chemical structure of SGG resulted in creating a more disordered system with DMPC than did SGC. This increased effect in disordering the lipid bilayers is likely due in part to the alkyl group in ether linkage at position *sn*-1 of SGG molecules. Examination of carbon-oxygen stretching vibrations at the interfacial region of the mixed liposomes revealed hydrogen bonding was disrupted due to the insertion of the co-existing lipid. This disruption of hydrogen bonding likely resulted from mutual shielding by either lipid of the other's hydrogen bonding partners (i.e., hydroxy groups on the sugar polar head of the sulfoglycolipids and α -hydroxy groups in the SGC system). The HP-FTIR study

of the methylene bending mode of mixed SGC+DMPC liposomes revealed the co-presence of the two lipids resulted in an increase in hydrocarbon chain orientational and conformational disorder of both lipid components. This result was of special interest since past studies were only able to deduce the possible interaction between a sulfoglycolipid and a phospholipid (Nabet et al., 1996). Through the use of HP-FTIR, our study provided an actual measure of the resulting disorder upon interaction of the hydrocarbon chains of SGC and a model phospholipid. Previous HP-FTIR studies in our laboratory revealed the correlation field splitting pressure of sodium salt form SGG was 3.3 kbar (Tupper et al., 1994) and the reported splitting pressure of DMPC was 3.2 kbar (Auger et al., 1988; Wong et al., 1988; Wong, 1994). The splitting pressure value of SGG and DMPC were so close to one another that repeating the same HP-FTIR approach used for SGC was not practical. Instead, in an attempt to study the effect of mixing SGG with DMPC we examined the CH₂ stretching modes of SGG liposomes versus SGG+deuterated DMPC at different temperatures. This thermotropic approach to studying membrane dynamics may be especially useful when investigating the interaction of sperm lipid components since spermatogenesis is temperature sensitive (Yanagimachi, 1994). Our thermotropic FTIR results on the hydrocarbon region of SGG liposomes versus SGG+DMPC liposomes again revealed mixing SGG with DMPC resulted in an increase in the disorder of SGG hydrocarbon chains.

Overall, the interaction between sulfoglycolipid and our model phospholipid can be described as increasing hydrocarbon chain disorder consistent with a disruption of hydrogen bonding at the interfacial region. Although our system was a very simple one, as compared

to the natural sperm plasma membrane, it was a first attempt to understand the parameters that control the dynamics of the complex sperm plasma membrane. Studies examining more complex and more physiologically representative systems must be conducted to determine whether the increase in sperm membrane fluidity observed upon capacitation (Wolf, 1986a) could result from similar membrane dynamics as observed between the lipids in this study.

A number of additional experiments should be performed in order to promote better understanding of the physiological roles of SGG and SGC. First, phospholipids that are found in the sperm plasma membrane should be used in place of DMPC. In the present study, DMPC was selected based on its availability in deuterated form and the extensive FTIR information available on its behaviour in model membrane systems. However, polyunsaturated, long acyl chain (e.g., C22:6) species of phosphatidylcholine and palmitoyloleoyl phosphatidylcholine may serve as more valuable and appropriate model phospholipids, as they are present naturally as main phosphatidylcholine molecular species on the sperm plasma membrane (Park and Lynch, 1992). Unfortunately, polyunsaturated, long acyl chain phosphatidylcholine and the deuterated form of this phospholipid and palmitoyloleoyl phosphatidylcholine are not readily available commercially. The lack of the deuterated form of these phospholipids makes it impossible to exclude the IR absorption of the methylene chains of the phospholipid in the mixed liposomes (SGG/SGC + phospholipid) from that of SGG/SGC methylene chains.

Previous results from our laboratory indicate that Ca^{2+} binding to SGG/SGC liposomes results in an increase in the disorder of the bilayer membranes (Tupper et al., 1992, 1994). It will be interesting to further this Ca^{2+} binding study with the mixed

liposomes of phospholipid and SGG/SGC. As well, the effect of the sulfate group of SGG and SGC could be further characterized through substituting these sulfoglycolipids with the parent glycolipids, GG and GC, in the model liposome systems. Results from such studies may elucidate the effects of SGG desulfation (which may occur during late capacitation or the acrosome reaction, Tanphaichitr et al., 1991) on the sperm plasma membrane function.

Furthermore, similar IR studies as described in this study should be performed with the mixed liposomes of various molar ratios of SGG/SGC: phospholipid. Our choice of a molar ratio of 2:3 was based on: 1) phosphatidylcholine, the major phospholipid of sperm plasma membranes, accounts for at least 30% (Brouwers et al., 1998); 2) SGG is present at ~25% of the lipid content in the sperm head anterior (B. Gadella, Utrecht University, personal communication); and 3) only at this molar ratio, a strong IR signal for the amide C=O group of the SGC molecules, which contain a variety of molecular subclasses, was observed. Nonetheless, the exact ratio of SGG to phospholipid at a specific location in the mammalian sperm head is not exactly known, and it will be informative to obtain IR results with various molar ratios of SGG to phospholipid.

"Fluidity" described in this thesis is based on FTIR studies, which detect vibrational and rotational movement of a structural group. This vibrational and rotational movement is very rapid and may not accurately represent membrane fluidity in a physiological sense. To be more precise, "fluidity" used throughout this thesis may rather reflect structural disorder. Therefore, other biophysical techniques, which have been used in membrane fluidity studies, should also be applied to determine the contribution of SGG to fluidity of model membranes or of sperm plasma membrane. These biophysical

techniques include differential scanning calorimetry, electron spin resonance and polarization spectroscopy, and fluorescence recovery after photobleaching (FRAP). Especially, FRAP has an advantage in giving fluidity data at a specific membrane domain of intact cells, and it has been successfully used in detecting changes of membrane fluidity of sperm at different capacitation stages (Wolf et al., 1998). Unlike FTIR which does not require any probe labeling, FRAP employs a fluorescent reporter probe that is inserted into the membrane bilayer during its incubation with the cells, and the diffusion of the fluorescent probe is calculated after laser induced photobleaching and recovery (Wolf et al., 1998). This fluorescent labeling may cause membrane perturbation and sometimes damage, as observed microscopically (N. Tanphaichitr, personal communication).

The results described in this thesis have provided a better understanding of the proposed physiological roles of SGG/SGC, particularly with respect to their influence on membrane fluidity. They should also form a foundation for future experiment approaches as described above. Determining how sperm membrane fluidity is modulated by SGG (as described in this thesis) will further our understanding of the mechanisms underlying sperm-egg interaction, since it is believed that an increase in sperm membrane fluidity is beneficial to the events of sperm-egg interaction, sperm acrosome reaction and sperm-egg plasma membrane fusion (Yanagimachi, 1994). Furthermore, our results related to SGC should be beneficial to better understanding of its role in maintaining the myelin structure (Murray and Narasimhan, 1990).

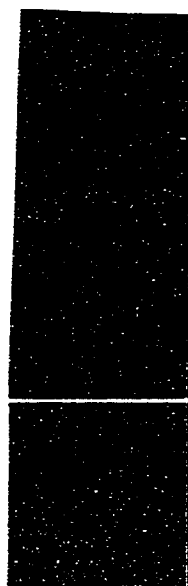
APPENDIX I

Azure A/H₂SO₄ staining is specific to the presence of a sulfate group (Kates, 1986).

SGG stained blue-white in lanes 1 and 2 which verified the presence of the sulfate group.

GG in lane 3 did not stain positive which indicated the absence of a sulfate group.

Figure I Thin layer chromatogram: SGG sodium salt (prepared for FTIR studies described in section 3.2) (lane 1); standard SGG ammonium salt (lane 2); and GG (lane 3). The lipids were separated using the solvent system $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (65:25:4, v/v/v). The lipids were then visualized by Azure A/ H_2SO_4 staining.



1 2 3

APPENDIX II

Table I

Negative FAB-MS minor ion peaks^a of purified ram testes SGG.

Identity of Ion Peaks ^b	Mass ^c		Relative Abundance (%) ^d
	Found	Calculated	
SGG with C14:0 fatty acid	767.5	767	8.3
SGG with C14:1 fatty acid	765.5	765	3.3
SGG with C15:0 alcohol	779.5	779	3.2
SGG with C 15:1 alcohol	779.5	779	3.2
SGG with C17:0 alcohol	809.5	809	11.7
SGG with C18:0 fatty acid	823.5	823	9.3

^a These molecular subclasses were identified as minor components by GLC analysis (Tupper et al., 1994).

^b When not indicated otherwise, assume *sn*-2 acyl chain is C16:0 and *sn*-1 alcohol chain is C16:0.

^c The mass value in the first column (i.e., Found) represents the ratio of the mass of the ion to the charge on the ion (Pavia et al., 1979).

^d This value represents the percent relative abundance of a specific ion peak relative to the abundance of the base ion peak (corresponds to SO₃⁻NH₃⁺ with m/z of 97.0). This percent value reflects the stability of the molecular ion formed rather than the relative amount formed of the ion.

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