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THE EFFECTS OF DIVALENT CATIONS
ON THE DYNAMICS OF SULFOGALACTOSYLCERAMIDE AND
SULFOGALACTOSYLACYLALKYLGLYCEROL MULTILAYERS: A STUDY
USING THE TECHNIQUE OF HIGH PRESSURE FOURIER-TRANSFORM
INFRARED SPECTROSCOPY

by

Heather Susanne Tupper

Thesis submitted to the Department of Biochemistry in partial
fulfillment of the requirements for the degree of
Master of Science

University of Ottawa

Ottawa, Ontario, Canada

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This thesis is dedicated to my family.

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ABSTRACT

Sulfogalactosylacylglycerol (SGG) and sulfogalactosylceramide (SGC) are structurally related sulfatides that are found ubiquitously in the plasma membrane of male germ cells. SGG is present selectively in mammalian germ cells, whereas SGC is the main sulfatide in the germ cells of lower species, although it is present in small quantities in germ cells of certain mammalian species. Although their exact cellular function(s) are unknown, it is speculated that SGG/SGC may play a role in cation transport and sperm/egg binding.

Since Ca^{2+} is a cation that plays important roles in many fertilization related events (Yanagimachi, 1988), we have been interested in determining whether Ca^{2+} interacts with the negatively charged SGG/SGC and whether this interaction results in changes to the lipid dynamics. Furthermore, other divalent cations have been shown to have similar (Sr^{2+}) or counteracting (Mg^{2+}) effects to Ca^{2+} in various events related to mammalian sperm functions (Stock & Fraser, 1989; Rogers & Yanagimachi, 1976). Therefore, the lipid dynamic effects of Sr^{2+} and Mg^{2+} binding to SGG were compared to those resulting from Ca^{2+} binding. In this study, we used high pressure Fourier-transform infrared spectroscopy, a powerful technique that reveals information on different structural regions of the lipid molecule based on their specific infrared vibrational absorption modes. The study was performed using multilayer suspensions of the sulfatides.

Our results showed that all three divalent cations electrostatically interacted with the sulfate moiety of hydrated SGG, although with varying degrees of strength. Similarly

Ca^{2+} bound to the sulfate moiety of solid SGC. It was found that hydrated SGG- Na^+ was interdigitated whereas hydrated SGC- Na^+ was not. The divalent cations presumably crosslinked the sulfate groups of neighbouring lipid molecules. In the case of SGG, this crosslinking removed the interdigitation. With SGC, the crosslinking by Ca^{2+} weakened the hydrogen bonding of the interfacial region and the hydrocarbon chains became more disordered. Similar lipid dynamic effects of divalent cations were found in the SGG studies, with Mg^{2+} inducing the greatest chain disorder followed by Ca^{2+} then Sr^{2+} . An increase in chain disorder would increase the bilayer fluidity. Such a phenomenon may prove relevant to the changes of the sperm plasma membrane during fertilization-related event.

LIST OF TABLES:

TABLE	DESCRIPTION	PAGE
3.1	R_f values of nGC, α GC, mGC, SGC, and SGG separated on TLC.	30
3.2	Summary of standard FAME results from GLC analysis at 180°C.	35
3.3	Summary of SGG FAME results from GLC analysis at 180°C.	37
3.4	Summary of standard FAME results from GLC analysis at 215°C.	39
3.5	Summary of SGG FAME results from GLC analysis at 215°C.	41
3.6	Summary of standard MAGD results from GLC analysis at 215°C.	43
3.7	Summary of SGG MAGD results from GLC analysis at 215°C.	45
3.8	Overall molecular subclass composition of SGG.	46
3.9	Height intensities of δ' CH ₂ and δ CH ₂ bands and $I\delta'$ CH ₂ / $I\delta$ CH ₂ ratio (R) over increasing pressure for SGG-Na ⁺ .	76
3.10	Height intensities of δ' CH ₂ and δ CH ₂ bands and $I\delta'$ CH ₂ / $I\delta$ CH ₂ ratio (R) over increasing pressure for SGG-Ca ²⁺ .	77
3.11	Height intensities of δ' CH ₂ and δ CH ₂ bands and $I\delta'$ CH ₂ / $I\delta$ CH ₂ ratio (R) over increasing pressure for SGG-Mg ²⁺ .	79
3.12	Height intensities of δ' CH ₂ and δ CH ₂ bands and $I\delta'$ CH ₂ / $I\delta$ CH ₂ ratio (R) over increasing pressure for SGG-Sr ²⁺ .	80

TABLE	DESCRIPTION	PAGE
3.13	Height intensities of γ' CH ₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Na ⁺ .	87
3.14	Height intensities of γ' CH ₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Ca ²⁺ .	89
3.15	Height intensities of γ' CH ₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Mg ²⁺ .	91
3.16	Height intensities of γ' CH ₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Sr ²⁺ .	93

LIST OF FIGURES:

FIGURE	DESCRIPTION	PAGE
1.1	Schematic representation of (A) SGC with α -hydroxy fatty acid, (B) SGC with non-hydroxy fatty acid, and (C) SGG.	2
3.1	Thin-layer chromatogram of nGC, α GC, mGC, SGC, SGG and PC/PE.	29
3.2	The infrared spectra of (A) boar testis SGG in the frequency range 400-1800 cm^{-1} (from Ishizuka et al., 1973) and (B) ram testis SGG in the frequency range of 400-1800 cm^{-1} .	32
3.3	GLC chromatogram of standard FAMES at 180°C.	34
3.4	GLC chromatogram of SGG FAMES at 180°C.	36
3.5	GLC chromatogram of standard FAMES at 215°C.	38
3.6	GLC chromatogram of SGG FAMES at 215°C.	40
3.7	GLC chromatogram of standard MAGDs at 215°C.	42
3.8	GLC chromatogram of SGG MAGDs at 215°C.	44
3.9	The infrared spectra of solid SGC- Na^+ , SGC- Ca^{2+} , and mGC in the S=O asymmetric stretching region (1200-1240 cm^{-1}) at ambient pressure.	49

FIGURE	DESCRIPTION	PAGE
3.10	The infrared spectra of hydrated SGC- Na^+ , SGC- Ca^{2+} , and mGC in the C-O stretching region ($950\text{-}1020\text{ cm}^{-1}$) at ambient pressure.	51
3.11	The infrared spectra of solid SGC- Na^+ and SGC- Ca^{2+} in the O-H and N-H stretching region ($3100\text{-}3700\text{ cm}^{-1}$) at ambient pressure.	52
3.12	The infrared spectra of solid mGC, nGC, and α GC in the amide I C=O stretching region ($1600\text{-}1700\text{ cm}^{-1}$) at ambient pressure.	54
3.13	(A) The infrared spectra of hydrated SGC- Na^+ and SGC- Ca^{2+} in the amide I C=O stretching region ($1600\text{-}1700\text{ cm}^{-1}$). (B) The third-order Fourier-derivative spectra of hydrated SGC- Na^+ and SGC- Ca^{2+} in the amide I C=O stretching region, using a breakpoint of 0.2 in the Fourier domain.	55
3.14	Stacked contour plots of the infrared spectra of the CH_2 scissoring mode ($1430\text{-}1500\text{ cm}^{-1}$) of (A) hydrated nGC and (B) hydrated α GC. The numbers on the right of the plots are pressure values in kbar.	57
3.15	Stacked contour plots of the infrared spectra of the CH_2 scissoring mode ($1430\text{-}1500\text{ cm}^{-1}$) of mGC. The numbers on the right of the plots are pressure values in kbar.	58
3.16	Stacked contour plots of the infrared spectra of the CH_2 scissoring mode ($1430\text{-}1500\text{ cm}^{-1}$) of (A) hydrated SGC- Na^+ and (B) hydrated SGC- Ca^{2+} . The numbers on the right of the plots are pressure values in kbar.	60

FIGURE	DESCRIPTION	PAGE
3.17	Pressure dependence of the frequencies of the CH ₂ scissoring mode (1460-1480 cm ⁻¹) for (+) hydrated SGC-Na ⁺ , (○) hydrated SGC-Ca ²⁺ , and (●) hydrated mGC.	61
3.18	Pressure dependence of the frequencies of the CH ₃ bending mode (1377-1381 cm ⁻¹) for (+) hydrated SGC-Na ⁺ and (○) hydrated SGC-Ca ²⁺ .	62
3.19	(A) The infrared spectra of hydrated SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the S=O asymmetric stretching region (1200-1240 cm ⁻¹) at ambient pressure. (B) The Fourier-deconvolved spectra of hydrated SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the S=O asymmetric stretching region.	65
3.20	The infrared spectra of solid SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the S=O asymmetric stretching region (1200-1240 cm ⁻¹) at ambient pressure.	67
3.21	The infrared spectra of hydrated SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the S-O stretching region (780-860 cm ⁻¹) at ambient pressure.	68
3.22	The infrared spectra of hydrated SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the C-O stretching region (965-1015 cm ⁻¹) at ambient pressure.	69
3.23	The infrared spectra of hydrated SGG-Na ⁺ , SGG-Ca ²⁺ , SGG-Mg ²⁺ , and SGG-Sr ²⁺ in the ester C=O stretching region (1650-1750 cm ⁻¹) at ambient pressure.	71
3.24	Pressure dependence of the frequencies of the low-frequency component of the ester C=O stretching mode (1710-1720 cm ⁻¹) for (+) hydrated SGG-Na ⁺ , (○) hydrated SGG-Ca ²⁺ , (●) hydrated SGG-Mg ²⁺ , and (X) hydrated SGG-Sr ²⁺ .	73

FIGURE	DESCRIPTION	PAGE
3.25	Stacked contour plots of the infrared spectra of the CH_2 scissoring mode ($1430\text{-}1500\text{ cm}^{-1}$) of (A) hydrated SGG- Na^+ and (B) hydrated SGG- Ca^{2+} . The numbers on the right of the plots are pressure values in kbar.	75
3.26	Stacked contour plots of the infrared spectra of the CH_2 scissoring mode ($1430\text{-}1500\text{ cm}^{-1}$) of (A) hydrated SGG- Mg^{2+} and (B) hydrated SGG- Sr^{2+} .	78
3.27	Pressure dependence of the peak height intensity ratio R ($I_{\delta'\text{CH}_2}/I_{\delta\text{CH}_2}$) for (+) hydrated SGG- Na^+ , (O) hydrated SGG- Ca^{2+} , (●) hydrated SGG- Mg^{2+} , and (X) hydrated SGG- Sr^{2+} .	81
3.28	Pressure dependence of the frequencies of the CH_2 scissoring mode ($1460\text{-}1478\text{ cm}^{-1}$) for (+) hydrated SGG- Na^+ , (O) hydrated SGG- Ca^{2+} , (●) hydrated SGG- Mg^{2+} , and (X) hydrated SGG- Sr^{2+} .	83
3.29	Stacked contour plots of the infrared spectra of the CH_2 rocking mode in the frequency range of $675\text{-}840\text{ cm}^{-1}$ for hydrated SGG- Na^+ . Arrow marks correlation field splitting band, $\delta'\text{CH}_2$.	86
3.30	Stacked contour plots of the infrared spectra of the CH_2 rocking mode in the frequency range of $675\text{-}840\text{ cm}^{-1}$ for hydrated SGG- Ca^{2+} . Arrow marks correlation field splitting band, $\delta'\text{CH}_2$.	88
3.31	Stacked contour plots of the infrared spectra of the CH_2 rocking mode in the frequency range of $675\text{-}840\text{ cm}^{-1}$ for hydrated SGG- Mg^{2+} . Arrow marks correlation field splitting band, $\delta'\text{CH}_2$.	90
3.32	Stacked contour plots of the infrared spectra of the CH_2 rocking mode in the frequency range of $675\text{-}840\text{ cm}^{-1}$ for hydrated SGG- Sr^{2+} . Arrow marks correlation field splitting band, $\delta'\text{CH}_2$.	92

FIGURE	DESCRIPTION	PAGE
3.33	Pressure dependence of the height intensity ratio R' ($I_{\gamma'CH_2}/I''_{valley}$) for (+) hydrated SGG- Na^+ , (O) hydrated SGG- Ca^{2+} , (●) hydrated SGG- Mg^{2+} , and (X) hydrated SGG- Sr^{2+} .	94
3.34	Pressure dependence of the frequencies of the CH_3 bending mode ($1379-1383\text{ cm}^{-1}$) for (+) hydrated SGG- Na^+ , (O) hydrated SGG- Ca^{2+} , (●) hydrated SGG- Mg^{2+} , and (X) hydrated SGG- Sr^{2+} .	96
A1.1	The electromagnetic spectrum.	132
A1.2	Light absorption and emission by a molecule.	133
A1.3	A simple harmonic oscillation consisting of two masses, m_1 and m_2 .	135
A1.4	(A) Energy of a simple harmonic oscillator as a function of the distance between the two masses. (B) Energy of a diatomic molecule as a function of atomic separation.	136
A1.5	Stretching and bending vibration for AX_2 and AX_3 groups.	138
A1.6	A multilamellar vesicle.	147
A2.1	Schematic representation of methanolysis/acetylation reaction of SGG to obtain FAMES and MAGDs.	152
A3.1	Schematic diagram of a flame ionization detector.	155

LIST OF ABBREVIATIONS:

αGC	Galactosylceramide with α -hydroxy fatty acid
AS-A	Arylsulfatase A
DSC	Differential scanning calorimetry
FAME	Fatty acid methyl esters
GC	Galactosylceramide
GG	Galactosylacylalkylglycerol
GLC	Gas-liquid chromatography
HP FTIR	High pressure Fourier-transform infrared spectroscopy
IR	Infrared
MAGD	Monoalkyl glycerol diacetate
mGC	mixed Galactosylceramide: ~60% with α -hydroxy fatty acid and ~40% with non-hydroxy fatty acid
nGC	Galactosylceramide with non-hydroxy fatty acid
NMR	Nuclear magnetic resonance spectroscopy
PAP	3'-Phosphoadenosine 5'-phosphate
PAPS	3'-Phosphoadenosine 5'-phosphosulfate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
SGC	Sulfogalactosylceramide
SGC-Na⁺	Na ⁺ salt of SGC

SGC-Ca^{2+}	Ca^{2+} salt of SGC
SGG	Sulfogalactosylalkylglycerol
SGG-Na^+	Na^+ salt of SGG
SGG-Ca^{2+}	Ca^{2+} salt of SGG
SGG-Mg^{2+}	Mg^{2+} salt of SGG
SGG-Sr^{2+}	Sr^{2+} salt of SGG
SLIP1	Sulfolipid immobilizing protein 1
TLC	Thin-layer chromatography
ZP	Zona pellucida

CONTENTS

Acknowledgements	ii
Abstract	iii
List of Tables	v
List of Figures	vii
List of Abbreviations	xii
Contents	xiv
CHAPTER ONE	
Introduction	1
1.1 Sulfogalactosylceramide	1
1.2 Sulfogalactosylacylalkylglycerol	6
1.3 Events of Fertilization	12
1.4 Divalent Cations and Fertilization	13
1.5 The Hypothetical Influence of Ca^{2+} and Lipids on Membrane Function Related to Fertilization	15
1.6 Research Objectives	17
CHAPTER TWO	
Materials and Methods	20
2.1 Materials	20
2.2 Extraction and Purification of SGG	20
2.3 Purity Verification of SGG, SGC, and GC	21
2.4 Characterization of SGG Acyl and Alkyl Moieties	22
2.5 GLC Analysis	24
2.6 Removal of Endogenous Cations from SGC and SGG	24
2.7 Preparation of Multilamellar Bilayers	25
2.8 Spectroscopic Analysis	26
CHAPTER THREE	
Results	28
3.1 Characterization of SGG, SGC, and GC	28
3.2 Infrared Analysis of SGC and GC	47
3.2.1 Calcium Binding to the Negatively-Charged Sulfate Moiety	48
3.2.2 The Interfacial Region (Amide Group)	53
3.2.3 The Hydrocarbon Chain Structure	56

3.3 Infrared Analysis of SGG	63
3.3.1 Cation Interaction with the Negatively- Charged Sulfate Moiety	64
3.3.2 The Interfacial Region (Ester Group)	70
3.3.3 The Hydrocarbon Chain Structure	74
CHAPTER FOUR	
Discussion	97
4.1 Characterization of SGG, SGC, and GC	97
4.2 Infrared Analysis of SGC and GC	99
4.2.1 Calcium Binding to the Negatively- Charged Sulfate Moiety	99
4.2.2 The Interfacial Region (Amide Group)	101
4.2.3 The Hydrocarbon Chain Structure	105
4.3 Infrared Analysis of SGG	109
4.3.1 Cation Interaction with the Negatively-Charged Sulfate Moiety	109
4.3.2 The Interfacial Region (Ester Group)	112
4.3.3 The Hydrocarbon Chain Structure	115
4.4 Significance of Research Findings	122
APPENDIX ONE	
Theory of Infrared Spectroscopy	130
A1.1 Background Theory on Infrared Dispersion Spectroscopy	130
A1.2 Fourier-Transform Infrared (FTIR) Spectroscopy	139
A1.3 Fourier Analysis of Infrared Data	140
A1.4 High-Pressure Fourier-Transform Infrared Analysis	142
A1.5 Application of High-Pressure Fourier-Transform Infrared Spectroscopy to Lipids	144
APPENDIX TWO	
Methanolysis/Acetylation Reactions of SGG	152
APPENDIX THREE	
Theory of Gas-Liquid Chromatography	153
References	159

CHAPTER ONE

INTRODUCTION

1.1 Sulfogalactosylceramide

Sulfogalactosylceramide (SGC) is a sulfoglycosphingolipid that is localized in several animal tissues, including brain (Norton and Autilo, 1966), retina (Dreyfus, Pieringer, Farooqui, Harth, Rebel, and Sarlieve, 1978), kidney (Martensson, 1963), skeletal muscle (Sung, Esselman, and Sweeley, 1973), uterus (Mikami, 1991) and testes of birds, fish, reptiles, amphibians, and certain mammals including humans (Murray and Narasimhan, 1990).

The chemical structure of SGC is shown in figure 1.1A,B. In the literature, it has been referred to as sulfatide, galactosylceramidesulfate, sulfato-galactosylceramide, and cerebroside sulfate. It was first discovered in 1884 by Thudichum, who isolated a sulfur-containing lipid from the human brain and gave it the name sulfatide. However, the structure of SGC was not chemically characterized until 1933 by Blix who identified its constituents as sphingosine, fatty acid, galactose, and sulfate. The position of the sulfate was later determined to be an ester of the galactose 3-hydroxyl group (Yamakawa, Kiso, Handa, Makita, and Yokoyama, 1962; Stoffyn and Stoffyn, 1963).

Figure 1.1 - Schematic representation of (A) sulfogalactosylceramide (SGC) with α -hydroxy fatty acid, (B) sulfogalactosylceramide (SGC) with non-hydroxy fatty acid, and (C) sulfogalactosylacylalkylglycerol (SGG).

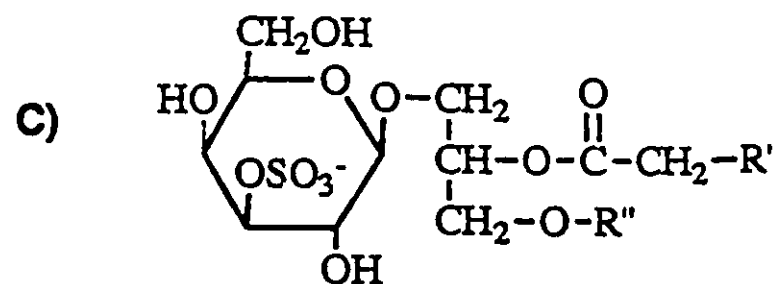
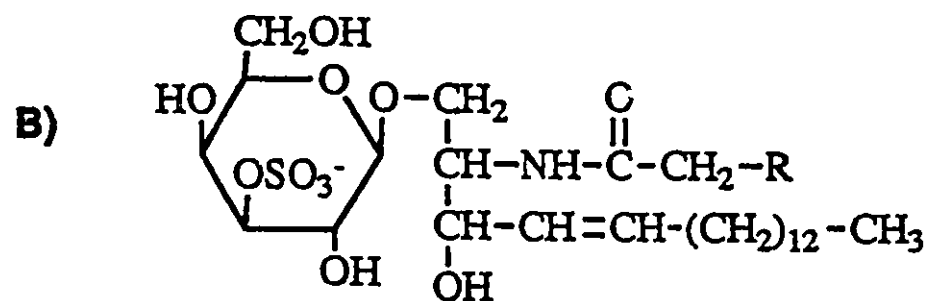
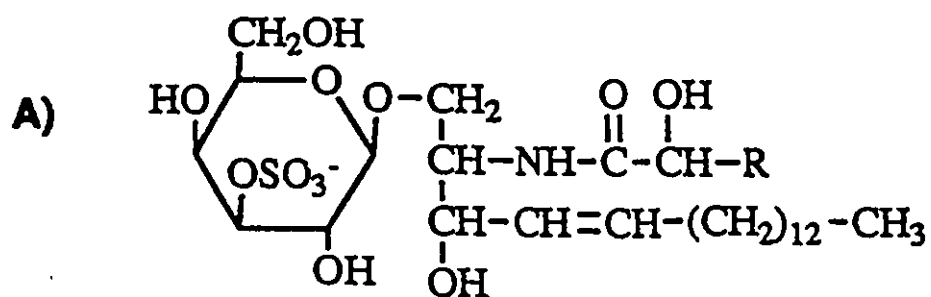


Figure 1.1

SGC is synthesized by sulfation of the 3-hydroxyl group of the galactose moiety of GC. The enzyme galactolipid sulfotransferase, which is localized in the Golgi apparatus, transfers sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to GC by the following reaction:



The transportation of SGC from the Golgi apparatus to the plasma membrane is not clearly understood. Herschkowitz, McKhann, Saxena, and Shooter (1968) have proposed that lipoproteins act as the vehicles for sulfatide transport in the cytosol. More recently, lysosomes have also been suggested as carriers of sulfatides (Burkart, Hofmann, Siegrist, Herschowitz, and Weismann, 1981; Burkart, Caimi, Siegrist, Herschokowitz, and Wiesmann, 1982).

The intramolecular and intermolecular hydrogen bonding of GC, the nonsulfated parental compound of SGC, has been extensively characterized using infrared spectroscopy and X-ray studies (Pascher, 1976; Pascher and Sundell, 1977; Jackson, Johnston, and Chapman, 1988). Crystalline GC participates in lateral hydrogen bonds between the amide group and neighbouring sugar head groups. The presence of an α -hydroxyl group in the GC fatty acid chain further increases the degree of intramolecular and intermolecular hydrogen bonding (Pascher and Sundell, 1977). A molecular species of SGC with an α -hydroxylated fatty acyl chain also exists (Figure 1.1A). A differential scanning calorimetry study by Boggs (1987) indicated that α -hydroxylation of the SGC and GC fatty acid increases stability of the gel phase, presumably due to additional

hydrogen bonds. Consequently, a higher proportion of α -hydroxylated to nonhydroxylated species of SGC and GC in the membrane would result in an increase in membrane stability concurrent with a decrease in membrane fluidity/permeability. Consistent with this is the observation that sphingolipids of membranes exposed to physical and chemical stress have a higher proportion of α -hydroxylated species (Pascher and Sundell, 1977). In contrast, Singh and colleagues have shown that α -hydroxylation of GC in gel phase phospholipid membranes leads to increased conformational disorder (Singh, Jarrell, Florio, Fenske, and Grant, 1992). The binding of divalent cations such as Ca^{2+} to the sulfate moiety of SGC may alter the degree of intermolecular and intramolecular hydrogen bonding, which would affect lipid and bilayer fluidity and function.

SGC, like all galactolipids, is present in the outer leaflet of the plasma membrane bilayer (Hakomori, 1981). While its cellular functions have not been clearly established, it has been implicated in cation transport, cell adhesion, immune recognition, toxin receptor and modulation of cell membrane structure (Curatolo, 1987; Farooqui, 1981; Karlsson, 1989; Karlsson, 1991). It has been suggested that SGC is involved in the active transport of Na^+ (Karlsson, Leffler, and Samuelsson, 1974). This suggestion is based on the finding that SGC is the major glycosphingolipid of the salt glands of eider duck and herring gull which have a high capacity for transporting sodium ions. Also, Ishizuka and Nakamura (1991) found that kidney epithelial cells cultured in high ionic strength medium contained higher levels of SGC than cells cultured in normal ionic strength medium. Benjamins and Dyer (1990) have reported that the binding of anti-GC

and anti-SGC antibodies by cultured oligodendrocytes induces the opening of Ca^{2+} channels, suggesting a role of these glycolipids in Ca^{2+} transport. Quinn and Sherman (1971) showed that SGC monolayers have a high affinity for Ca^{2+} , although direct involvement of SGC in the binding and/or transporting of Ca^{2+} into the cell has not yet been illustrated. Several studies have revealed that agglutination of red blood cells by the basement membrane glycoprotein laminin involves the high affinity binding of sulfatide to laminin (Roberts, Rao, Magnani, Spitalnik, Liotta, and Ginsburg, 1975; Ginsburg and Roberts, 1988; Roberts and Ginsburg, 1988). Also, recent studies have implicated SGC and GC as receptors for the human immunodeficiency virus in oligodendrocytes and Schwann cells (Harouse, Bhat, Spitalnik, Laughlin, Stefano, Silberberg, and Gonzalez-Scarano, 1991; Bhat, Spitalnik, Gonzalez-Scarano, and Silberberg, 1991). The results of these studies suggest that sulfatides play a physiological role in cell adhesion. Several studies have speculated that in the brain, SGC may be part of the opiate receptor (Cho, Cho, and Loh, 1976; Loh, Law, Ostwald, Cho, and Way, 1978). Also, the high levels of SGC found in uterine epithelial cells during diestrus suggest that it may play a role in embryo implantation (Kubushiro, Kojima, Mikami, Nozawa, Iizuka, Iwamori, and Nagai, 1989; Mikami, 1991). Work by Hakomori (1983) has implicated glycosphingolipid involvement in cell interaction and differentiation, in cell growth control and in oncogenic transformation.

With respect to nervous tissue, SGC is found in high levels in the myelin sheath. It is synthesized during synaptogenesis and is thought to stabilize the compact structure of myelin through its electrostatic interactions with myelin proteins (Farooqui, 1981;

Burkart, Caimi, Siegrist, Herschkowitz, and Wiesmann, 1982). SGC can be desulfated by the lysosomal enzyme, cerebroside sulfatase (van der Pal, Klein, van Golde, and Lopes-Carbozo, 1990). A deficient cerebroside sulfatase activity is the cause of a genetically determined metabolic disorder, termed metachromatic leukodystrophy (Richardson and Adams, 1980). Because of deficient activity of cerebroside sulfatase, SGC accumulates excessively in many organs, especially brain and kidneys. The excessive accumulation of SGC eventually leads to breakdown of myelin, suggesting that its turnover is necessary for the structural stabilization of myelin (Richardson and Adams, 1980; Burkart et al., 1982).

1.2 Sulfogalactosylacylalkylglycerol

Sulfogalactosylacylalkylglycerol (SGG) is a glycolipid that is found only in the testes, spermatozoa, and brains of mammals such as rat, mouse, guinea pig, rabbit, boar, and human (Farooqui, 1981). SGG is the main glycolipid in testes (~5-8% of total lipid, ~90% of glycolipid), however, in the brain it is present only in small amounts (~0.2% of total lipid) (Ishizuka and Yamakawa, 1985). According to Gadella et al. (1992), a freshly ejaculated boar sperm cell contains approximately 1.8×10^8 SGG molecules. The chemical structure of SGG is shown in figure 1.1C. It is also referred to as seminolipid, sulfatide, and sulfatoxygalactosylacylalkylglycerol.

SGG was not discovered and characterized until two decades ago in 1972 by Kornblatt, Schachter, and Murray. Ishizuka, Suzuki, and Yamakawa (1973) reported its discovery around the same time. Upon isolation of the major glycolipid of rat testes,

Kornblatt et al. (1972) revealed through degradation studies the presence of chmyl alcohol, palmitic acid, galactose, and sulfate in equimolar amounts. The alkyl and acyl hydrocarbon chains were characterized as being predominantly (>85%) C16:0. Boar testis SGG was isolated by Ishizuka et al. (1973) and was also characterized as having equimolar amounts of fatty acid (>90% C16:0), glyceryl ether (>80% C16:0), galactose, and sulfate. IR and NMR studies indicated the presence of a β -anomeric configuration of the galactopyranoside, the presence of the sulfate of the 3'-position of the galactose moiety, and that the O-acyl group was attached to the C-2 of the glycerol. Based on the work of Ishizuka et al. (1973) and Alvarez, Storey, Hemling, and Grob (1990), the structure of SGG has been characterized as 1-O-alkyl-2-O-acyl-3-O-D-(3'-sulfo)galactopyranosyl-*sn*-glycerol (Figure 1.1C).

SGG is present only in the testis and brain of several mammalian species. It is neither found in other tissues nor the testis and brain of other vertebrate species. On the other hand, SGC has been found to be the major glycolipid in the testis and brain of lower vertebrates species, although it is also present in small quantities along with SGG, in germ cells of some mammalian species such as human (Murray and Narasimhan, 1990). The biological significance of these species differences in the presence of SGC and SGG is not known. It has been speculated that it may relate to subtle differences in testicular temperature (Murray and Narasimhan, 1990). The fact that the testes and spermatozoa of most animals preferentially contain either, or both of these sulfoglycolipids, suggests that SGC and SGG may play an important role in sperm function.

Galactolipid sulfotransferase may be responsible for both SGG and SGC biosynthesis from GG (the non-sulfate parental compound of SGG) and GC, respectively. Substrate competition studies demonstrated a clear competition between GC and GG indicating that galactolipid sulfotransferase acts on both galactosphingolipids and galactoglycerolipids (Knapp, Kornblatt, Schachter, and Murray, 1973). In maturing rat testis, the activity of PAPS-galactolipid sulfotransferase has been shown to decrease markedly, to about a third of the maximal level by 51 days after birth (Kornblatt, Knapp, Levine, Schachter, and Murray, 1974). Lingwood (1985c) found that the decrease in enzyme activity may be mediated by an inhibitor. In rat testis, the inhibitor appears to be substrate and tissue specific; it is most effective in preventing sulfation of GG and less so with respect to GC. In contrast, the activity of the sulfotransferases of kidney and brain was not affected by this inhibitor (Lingwood, 1985c).

SGG is synthesized in zygotene and pachytene spermatocytes, being an early marker of male germ cell differentiation. 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, 1985a). Furthermore, SGG is stable during capacitation (the process which prepares the sperm for egg penetration) (Tanphaichitr, Smith, and Kates, 1990) and turns over only when sperm become senescent (Kornblatt, 1979; Tanphaichitr et al., 1990). This implies a continuing putative function for SGG during sperm development, maturation, and sperm-zona pellucida interaction.

Both SGG and SGC serve as natural substrates of arylsulfatase-A (AS-A), an

enzyme that removes sulfate from the sugar head group. In boar semen, there are two subtypes of arylsulfatase-A, an intracellular AS-A located in the sperm acrosome, and an extracellular AS-A, which is secreted by the seminal vesicles into the seminal plasma during ejaculation (Gadella, Colenbrander, and Lopes-Cardozo, 1991; Gadella, Colenbrander, van Golde, and Lopes-Cardozo, 1992). According to studies by Gadella and colleagues, desulfation of SGG is a post-ejaculatory event, since SGG is likely located exclusively at the outer leaflet of the plasma membrane and is not exposed to AS-A until after ejaculation. Removal of the negatively charged sulfate group from SGG would most likely affect its interactions with other molecules and thus the function(s) of SGG. Moreover, the reaction product of the AS-A desulfation of SGG, GG, can aggregate via enhanced intermolecular hydrogen bonding (Sen, Mannock, Collins, Quinn, and Williams, 1983) and may have potent fusogenic properties which promote the hexagonal II state, thought to be a transitional state that induces membrane fusion of phospholipids (Cullis and de Kruijff, 1978; Gadella et al. 1991, 1992). The desulfation of SGG to GG may enhance the sperm acrosome reaction, which involves fusion of the sperm plasma membrane and the outer acrosomal membrane (see below).

The exact function(s) of SGG on the sperm plasma membrane is unknown, although there is circumstantial evidence that SGG and/or GG may play a role in sperm-egg binding and fusion (Lingwood, 1986; Lingwood et al., 1990; Tanphaichitr, Smith, Mongkolsiririkieart, Gradil, and Lingwood, 1993). Similar to other glycolipids and SGC (see above), SGG present on the sperm periacrosomal plasma membrane may play a role in cellular adhesion (i.e., binding of sperm to the zona pellucida of the egg) and ion

transport (Curatolo, 1987). Although no direct studies on the role of sperm SGG in sperm-zona pellucida binding have been described, several lines of evidence implicate the significance of SGG in fertility. A study by Tsuji and colleagues revealed the presence of sperm immobilizing antibodies in the sera of some infertile women. These antibodies react with the 3-O-sulfogalactose residue of SGG on the sperm membrane, suggesting that SGG may play an important role in fertilization (Tsuji, Fukuda, Iuchi, Ishizuka, and Isojima, 1992). Purified SGG incubated with *Mycoplasma pulmonis*, a microorganism known to cause infertility in rodents, has been shown to be degraded by desulfation and deacylation into GG, lysoSGG, and lysoGG (Lingwood, Schamayr, and Quinn, 1990). In fact, both SGG and SGC have been shown to be specifically recognized by all mycoplasmas involved in animal infertility (Lingwood, Quinn, Wilenskyy, Nutikka, Ruhnke, and Miller, 1990). Thus both SGG and SGC may serve as cell membrane receptors for mycoplasmas. Mycoplasma binding to the sperm has been shown to result in subsequent interference in sperm/egg receptor recognition (Busolo and Zanchetta, 1984).

Like other sulfated glycoconjugates (Holt, Krivan, Gasic, and Ginsburg, 1989), SGG has been shown to possess a specific binding protein. The ~68-kDa evolutionarily conserved germ cell plasma membrane protein, termed sulfoglycolipid immobilizing protein (SLIP1), has been shown to bind specifically to SGG *in vitro*, and to a lesser extent to SGC (Law, Itkonen, and Lingwood, 1988). Moreover, ATP has been shown to bind to SLIP1. The ATP-binding properties of SLIP1 have allowed for its crude extraction from rodent germ cells by an ATP-containing solution (Law et al., 1988). In

a double-labelling indirect immunofluorescence experiment, Lingwood (1986) used polyclonal rabbit anti-SLIP1 and monoclonal mouse anti-SGG to demonstrate colocalization of SLIP1 and SGG on rat spermatogenic cells. In rat caput sperm, SGG and SLIP1 were shown to be colocalized on the concave surface of the anterior head region and the midpiece. In rat cauda sperm, however, while most of SGG was found to remain in the same area as in caput sperm, SLIP1 was shown to be localized on the apical ridge of the sperm head (Lingwood, 1986). In contrast, indirect immunofluorescence labelling of mouse caudal sperm revealed that both SLIP1 and SGG appear in the same region of the sperm head, although their distribution patterns were not identical (Tanphaichitr et al., 1993). SLIP1 was distributed uniformly over the acrosomal region, including the apical ridge. SGG appeared in the acrosomal region as well, but anti-SGG staining at the apical ridge region of the sperm head was much lower in intensity in ~50% of the fixed sperm. The remaining 50% showed SGG localization either at the posterior acrosomal region that bordered the post acrosomal area. SGG was found to be present in the midpiece of all mouse and rat sperm, whereas SLIP1 was absent in this area (Lingwood, 1986; Tanphaichitr et al., 1993). All of these observations suggest that SLIP1 and SGG are partially or fully segregated in mouse and rat caudal sperm. SLIP1 is also found in mammalian brain cells and oocytes where it has been shown to retain the sulfogalactolipid-binding characteristics of rat testicular SLIP1 (Law et al., 1988). The localization of SLIP1 on the apical ridge of the sperm head, the initial zona pellucida binding site of sperm, suggests that SLIP1 may be involved in sperm-zona pellucida binding. Studies in our laboratory have supported this

hypothesis using both *in vivo* and *in vitro* approaches (Tanphaichitr, Tayabali, Gradil, Juneja, Leveille, and Lingwood, 1992; Tanphaichitr et al., 1993). No conclusive results have been obtained regarding the role of SGG in sperm-zona pellucida binding due to the lack of a monospecific IgG antibody. It is possible that SGG might influence SLIP1 function directly or indirectly, and in turn, sperm functions.

1.3 Events of Fertilization

Mature mammalian sperm, when ejaculated, are not capable of fertilizing eggs. They first must undergo capacitation, an event that involves physiological and functional changes that render the spermatozoa capable of fertilization. Capacitation normally takes place in the genital tract of the estrous female, although very little is known about the *in vivo* conditions or factors directly controlling the event (Yanagimachi, 1988). Capacitation can also be induced *in vitro* using a supplemented defined medium (Yanagimachi, 1988). Capacitation prepares the sperm for the acrosome reaction, an exocytotic event discussed below. In general, the various modifications that accompany capacitation include: 1) a decrease in the cholesterol:phospholipid ratio which results in an increase in membrane fluidity; 2) a modification of protein and sugar residues which probably results in the removal of inhibitory components and the exposure of egg receptors; 3) an elevated level of membrane-fusogenic lysophospholipids due to an increase in phospholipase A2 activity and a subsequential influx of Ca^{2+} ; 4) a higher adenylate cyclase activity; 5) a conversion of proacrosin to acrosin; and 6) an acquisition of hyperactivated motility patterns (Saling, 1989; Go and Wolf, 1983; Wolf, Hagopian,

and Ishijima, 1986; Koehler, 1981; Fraser, 1990; Langlais and Roberts, 1985; Parrish and Polakoski, 1979; Burkman, 1990). There are no morphological changes related to capacitation, in contrast to the acrosome reaction. The acrosome is a membrane-bound, cap-like structure located in the anterior portion of the sperm head, which contains a large array of hydrolytic enzymes, including arylsulfatase-A (Yanagimachi, 1988). During the acrosome reaction, the sperm plasma membrane fuses with the outer acrosomal membrane. This results in membrane vesiculation and finally shedding of the acrosomal contents. Presumably, the released hydrolytic enzymes serve to digest the cumulus matrix and zona pellucida of the egg (Yanagimachi, 1988).

In several mammals, it has been demonstrated that the zona pellucida is one of the agonists that induces the sperm acrosome reaction (Bleil and Wassarman, 1983; Cherr, Lambert, Meizel, and Katz, 1986; O'Rand and Fisher, 1987; Cross, Morales, Overstreet, and Hanson, 1988; Florman and First, 1988). Also, cumulus cells and progesterone entrapped in the follicular fluid have been shown to be acrosome reaction-inducing agonists (Blackmore, Beebe, Danforth, and Alexander, 1990; Meizel and Turner, 1991; Thomas and Meizel, 1989). Sperm exposure to any of these agonists leads to a sharp rise in intracellular Ca^{2+} levels, which subsequently triggers the acrosome reaction (Kopf and Gerton, 1990).

1.4 Divalent Cations and Fertilization

The presence of extracellular Ca^{2+} appears to be necessary for various activities of mammalian sperm. Extracellular Ca^{2+} present in the sperm culture media has been

shown to be required for capacitation, spontaneous acrosome reaction, hyperactivated motility, sperm-egg binding, and sperm-egg fusion (Fraser, 1987; Saling, Storey, and Wolf, 1978; Singh, Babcock, and Lardy, 1978; Yanagimachi, 1982; Yanagimachi and Usui, 1974). Among several divalent cations tested, such as Ba^{2+} , Mg^{2+} , and Sr^{2+} , only Sr^{2+} effectively mimicked Ca^{2+} in supporting capacitation and initiation of the acrosome reaction, but was less effective in promoting completion of the acrosome reaction (Stock and Fraser, 1989). In contrast, Ba^{2+} and Mg^{2+} were ineffective in substituting for Ca^{2+} with respect to capacitation and the acrosome reaction. Moreover, it has been demonstrated that Mg^{2+} counters the action of Ca^{2+} in regard to capacitation (Yanagimachi and Usui, 1974; Rogers and Yanagimachi, 1976). Roldan and Harrison (1989) have determined that three Ca^{2+} -dependent events can be distinguished during the processes leading to the acrosome reaction. The first event is the phosphoinositide breakdown. The two other events follow the first, one in which Sr^{2+} can substitute for Ca^{2+} and the other in which Sr^{2+} cannot. There is no indication as to what these two events may be; however, it is speculated that one may be Ca^{2+} -dependent membrane fusion. Yanagimachi and Usui (1974) have shown that Sr^{2+} also effectively supports the hyperactivated motility that provides the spermatozoa with a strong thrusting power, which is beneficial for sperm migration and sperm passage through the egg investments and zona pellucida (Yanagimachi, 1988).

The mechanism(s) of how Ca^{2+} influences the sperm functions are not exactly known, although it is probably a part of the signal transduction cascade that ends with an exocytotic event, the acrosome reaction. It has been shown that Ca^{2+} is present on

the sperm surface, although its binding molecule(s) is unknown (Ruknudin, 1989). Many studies have shown that increased permeability of Ca^{2+} coincides with increased membrane fluidity occurring during sperm capacitation (Babcock, Singh, and Lardy, 1979; Babcock, Stamerjohn, and Hutchinson, 1978). Voltage-gated Ca^{2+} channels have been suggested to play a role in Ca^{2+} influx during the acrosome reaction (Babcock and Pfeiffer, 1987; Florman, Corron, Kim, and Babcock, 1992). In contrast, Cox, Cambell, and Peterson (1991) could find no evidence of voltage-gated Ca^{2+} channels in boar sperm plasma membrane. However, their study did confirm the presence of channels capable of conducting divalent cations in the sperm plasma membrane. Seminal vesicle proteins, termed caltrin proteins, have been shown to regulate Ca^{2+} transport across the spermatozoal membrane (Coronel and Lardy, 1992; Lardy, San Agustin, and Coronel, 1988).

Both capacitation and the acrosome reaction involve membrane alterations that promote fusion. At the molecular level, this involves changes in membrane fluidity and the formation of specialized regions that may potentially represent sites of fusion. The fact that extracellular Ca^{2+} must be present to induce capacitation and the acrosome reaction, even when exogenous fusogenic lysophospholipids are supplied, suggests that Ca^{2+} plays a direct role in membrane fusion (Papahadjopoulos, 1978).

1.5 The Hypothetical Influence of Ca^{2+} and Lipids on Membrane Function Related to Fertilization

Studies performed on intact cells and isolated plasma membranes indicate that a

specific interaction occurs between Ca^{2+} and negatively charged sites on surface membranes (Gordon and Sauerheber, 1982). The fact that Ca^{2+} has been shown to be present on the sperm surface (Ruknudin, 1989) invites the suggestion that SGC and SGG may serve as "traps" of Ca^{2+} on the sperm surface. Ca^{2+} -induced membrane structural alterations have been well characterized in model membrane experiments. In general, Ca^{2+} -binding to acidic lipids increases the surface pressure, neutralizes the surface charge, and alters membrane fluidity (Gordon and Sauerheber, 1982). Moreover, Ca^{2+} may complex acidic lipids to membrane proteins and influence the lateral distribution of the proteins in the plane of the membrane bilayer (Gordon and Sauerheber, 1982). The binding of SGG and SLIP1 may be an example of such an interaction. It is possible that Ca^{2+} binding to SGG may influence the lateral distribution of SLIP1 in the sperm membrane.

The Ca^{2+} -induced effects on membrane enzyme activity may be exerted by direct Ca^{2+} -binding to specific protein sites, alteration of membrane surface charge, and change in the membrane fluidity. The enzyme activity of membrane-associated proteins may also be altered by Ca^{2+} -induced structural perturbations. Furthermore, enzyme function may also be affected via the linkage of acidic lipids to integral proteins by divalent cations. Finally, discrete lipid domains may be induced by Ca^{2+} such that proteins are segregated into restricted regions and protein-protein interactions are promoted (Gordon and Sauerheber, 1982). Such phenomena lend credence to the idea that Ca^{2+} and lipids play a direct role in membrane fusion, receptor recognition, and redistribution of membrane proteins.

SGG may be involved in membrane fusion for the following reasons. Firstly, the alkyl ether linkage at the *sn*-1-position of glycerol is thought to promote bilayer interdigitation (Ruocco, Siminovitch, and Griffin, 1985; Slater and Huang, 1988). Bilayer interdigitation results in an increased surface area and thus a reduced charge density per unit area and a decreased hydration pressure, conducive of membrane fusion (Kinnunen, 1992). Secondly, the negatively charged sulfate moiety of SGG may bind to Ca^{2+} . Ca^{2+} binding to acidic membrane lipids appears to be one of the critical determinants of membrane fusion (Papahadjopoulos and Poste, 1975; Papahadjopoulos, Nir, and Düzgünes, 1990). Thirdly, the sugar hydroxyl groups can provide hydrogen bonding sites for cross interaction with opposing bilayers containing the receptors of hydrogen bonding (Boggs, 1984; Boggs, 1987). Finally, the hydrocarbon chains of SGG can interact with the hydrophobic moieties of other lipids (Cullis and de Kruijff, 1978; Slater and Huang, 1989).

1.6 Research Objectives

The presence of SGC and SGG on the sperm plasma membrane may serve as potential "traps" of Ca^{2+} on the sperm surface. Given the predominant role Ca^{2+} plays in fertilization and its ability to induce membrane structural perturbations, we asked the question of whether Ca^{2+} binding to SGC and SGG multilayers would change their lipid dynamics. The fluidity of SGC/SGG domains may influence the lateral distribution on the sperm surface of SLIP1, which has been shown to play a role in sperm-zona pellucida binding (Tanphaichitr et al., 1992, 1993). Therefore, this study was initiated to

investigate the putative binding of Ca^{2+} to SGC/SGG and also the consequential effects on the lipid dynamics.

As a prelude to a study using intact sperm, this study was conducted using SGG and SGC multilayer suspensions. Specifically, the purpose of this study was to determine whether Ca^{2+} interacts with the negatively charged sulfate group of SGC/SGG and any effects the interaction may have on the hydrogen bonding of the interfacial region and finally the dynamics of the lipid hydrocarbon chains. Furthermore, given the ambiguous roles of Mg^{2+} and Sr^{2+} in fertilization and membrane function, this study also compared their interaction with SGG to that of Ca^{2+} . Specifically, we were interested in determining whether all three divalent cations interact with the negatively charged sulfate (and to what degree) and any effects they may have on the hydrogen bonding of the interfacial region and the dynamics of the hydrocarbon chains. SGC used in this study is a mixture of α -hydroxy and non-hydroxy fatty acids. Therefore, the study also determined the structural and dynamic effects of fatty acid α -hydroxylation, by comparison of the α -hydroxy GC (α GC), non-hydroxy GC (nGC), and mGC (a mixture of α GC and nGC) infrared spectra.

The technique of high-pressure Fourier-transform infrared (HP FT-IR) spectroscopy was used in this study. HP FT-IR spectroscopy is a powerful technique that reveals structural information of different regions of the lipid molecule based on its infrared vibrational absorption modes (see Appendix One). The application of HP FT-IR spectroscopy to model membranes has been well-studied and established (Auger, Jarrell, Smith, Siminovitch, Mantsch, and Wong, 1988; Auger, Smith, Mantsch, and Wong,

1990; Tupper, Wong, and Tanphaichitr, 1992; Wong, 1984, 1987; Wong and Mantsch, 1988, 1988).

Several absorption modes were analyzed, including: the S=O asymmetric stretching mode, the S-O stretching mode, the C-O stretching mode of the ester-linked sulfate group, the amide I mode (SGC, GC), the ester C=O stretching mode (SGG), the O-H stretching mode, the CH₂ scissoring mode, the CH₂ rocking mode, and the CH₃ bending mode. By comparing the frequencies, intensities, bandwidths, and barotropic behaviour of these absorption modes, the lipid dynamic effects of the divalent cations and fatty acid α -hydroxylation on the sulfatides were determined.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

SGC (containing 1.72% Na^+ , 1.07% Ca^{2+}) ($\sim 60\%$ with α -hydroxy fatty acid and $\sim 40\%$ with non-hydroxy fatty acid); three molecular species of GC (Ca^{2+} content not determined by manufacturer): type I (with α -hydroxy fatty acid (α GC)), type II (non-hydroxy fatty acid) (nGC), and mixed GC ($\sim 60\%$ with α -hydroxy fatty acid and $\sim 40\%$ with non-hydroxy fatty acid) (mGC); 1-O-hexadecyl-*sn*-glycerol; 1-O-octadecyl-*sn*-glycerol; phosphatidylethanolamine (PE); phosphatidylcholine (PC); D_2O (99.9 atom %); tris-(hydroxymethyl)aminomethane hydrochloride (Tris); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ were purchased from Sigma Chemical Co. (St. Louis, MO). High-purity natural crystalline α -quartz was obtained from Karl Lambrecht Corp. (Chicago, IL). Silica gel thin-layer chromatography (TLC) plates (250 μm layer, 20 x 20 cm) were purchased from Whatman (Kent, England). Bio-Sil A Silica (bead size 150-300 μm) for adsorption column chromatography was obtained from Bio-Rad (Richmond, CA). The glass columns for GLC were packed with 10% SP 2330 (Supelco, PA) on 100/120 Chromosorb W AW (John Mansville, NY).

2.2 Extraction and Purification of SGG

Lipids were extracted from the testes using the method of Bligh and Dyer (1959), as modified by Kates (1986). A pair of ram testes (~ 1 Kg) were generously provided

by Dr. Paul Fiser, CFAR. Each testis was homogenized in ~ 1500 mL of MeOH:CHCl₃ (2:1). The homogenate was incubated for 2 h at 4°C, then suction-filtered, using a Buchner funnel. The ratio of MeOH:CHCl₃:H₂O in the single phase filtrate was 2:1:0.8. Addition of equal volumes of CHCl₃ and H₂O to the single phase filtrate to give a final ratio of CHCl₃:MeOH:H₂O (1:1:0.9) resulted in formation of two phases. The mixture was transferred to a separatory funnel and left standing overnight at room temperature to allow complete separation of the two phases. The lower CHCl₃ phase, containing lipids, was removed, diluted with benzene (10%) and concentrated on a rotoevaporator.

Extracted lipids were separated by adsorption column chromatography using Bio-Sil A silica. Elution of the lipids was carried out using organic solvents in the following order: CHCl₃, 10 column volumes; 1:1 CHCl₃:acetone, 3 column volumes; acetone, 15 column volumes; and MeOH, 10 column volumes. Lipid contents of each fraction were assessed by ascending TLC using a solvent system that effectively separates glycolipids from other lipids, CHCl₃:MeOH:H₂O (65:25:4 v/v) (Levine, Bain, Narasimhan, Palmer, Yates, and Murray, 1976). The thin-layer chromatogram was air dried and glycolipid spots were detected by staining with a mixture of α -naphthol and 1:1 H₂SO₄:H₂O, followed by charring to detect standard lipid spots. Fractions containing SGG were pooled, dried, and precipitated in acetone (Kates, 1986).

2.3 Purity Verification of SGG, SGC and GC

According to information from Sigma Chemical Co., SGC and all three types of

GC were extracted from bovine brain. The purity of SGC, mGC, nGC, and α GC was 99%, 99%, 98%, and 97%, respectively. We confirmed their purity and that of SGG by ascending thin-layer chromatography (TLC) using the solvent system of CHCl_3 -MeOH- H_2O -conc. ammonia (65:25:3:1 v/v) (Kornblatt, 1979). Phosphatidylethanolamine and phosphatidylcholine were cochromatographed as lipid standards. Glycolipids were detected by their sugar staining with α -naphthol, whereas all lipids, which were exposed to H_2SO_4 , were detected by charring (Kates, 1986).

2.4 Characterization of SGG Acyl and Alkyl Moieties

The proportions of the acyl and alkyl subclasses of SGG were determined by using the modified method of Vishnubhatla, Kates, and Adams (1988) that involves: 1) acid methanolysis of SGG followed by Bligh and Dyer (1959) lipid extraction of fatty acid methyl esters (FAMES), which are derived from the acyl chain subclasses and, 2) subsequent acetylation to convert the lysoalkyl-SGG to a monoalkyl glycerol diacetate (MAGD) (see appendix two for schematic diagram of reaction). The proportions of SGG acyl and alkyl subclasses were determined through gas-liquid chromatography (GLC) analysis of the resulting FAMES and MAGDs (see appendix three for GLC theory).

Acid methanolysis of SGG involved the following procedure (Vishnubhatla et al., 1988): 1-2 mg of SGG was dissolved in 2 mL of 0.6 N HCl in MeOH and incubated at 75° for 2 h in a screw capped (Teflon-lined) test tube. After addition of 0.2 mL H_2O and 4 mL hexane, followed by vortexing, the upper hexane phase was removed and the lower phase was washed three times with hexane. The lower phase contained lysoalkyl-

SGG whereas SGG FAMES were partitioned into the hexane phase. The pooled hexane phases were concentrated under a stream of nitrogen and the residual lipids (SGG FAMES) were made up to a known volume in CHCl_3 and analyzed by GLC at 180°C and 215°C . SGG FAME peaks were identified by comparison to the relative retention times of standard FAMES 14:0, 16:0, 16:1, 18:0, and 18:1 that were kindly provided by Dr. M. Kates, University of Ottawa.

Conversion of the lysoalkyl-SGG to monoalkyl glycerol diacetates (MAGD) was accomplished by acetylation as follows. To the lower $\text{MeOH:H}_2\text{O}$ (2:0.2) phase, 2 mL of CHCl_3 and 1.6 mL of H_2O were added, followed by centrifugation (1700 rpm, 1 min) to separate the resulting two phases. The lower CHCl_3 phase was removed, and the upper phase was washed three times with CHCl_3 . The pooled CHCl_3 phases were diluted with benzene ($\sim 10\%$) and concentrated on a block heater ($\sim 40^\circ\text{C}$) under a N_2 stream. The residual lipids (lysoalkyl-SGG) were acetylated with 1 mL of glacial acetic acid:acetic anhydride (3:2) at room temperature for 72 h in a screw-capped (Teflon-lined) test tube. The solution was then heated to $\sim 120^\circ\text{C}$ for 2-3 h. Likewise, control MAGDs were prepared by acetylating of 1 mg each of 1-O-hexadecyl-*sn*-glycerol (16:0) and 1-O-octadecyl-*sn*-glycerol (18:0) with 1 mL each of glacial acetic acid:acetic anhydride (3:2) at 120°C for 2-3 h in a screw-capped (Teflon-lined) test tube. To each of the three cooled test tubes, 0.5 mL of H_2O and 4.5 mL of MeOH were added to form the corresponding MAGDs from the acetylated lysoalkyl-SGG, 1-O-hexadecyl-*sn*-glycerol, and 1-O-octadecyl-*sn*-glycerol. The resulting MAGDs were extracted with 3-4 portions (5 mL) of petroleum ether, evaporated under a stream of N_2 , and dried in a

desiccator over KOH *in vacuo*. The dried residual MAGDs were then made up to a known volume of CHCl_3 and analyzed by GLC at 215°C. SGG MAGD peaks were identified by comparison to the relative retention times of the standard MAGDS.

2.5 GLC Analysis

GLC analysis was performed on a PYE Unicam GLC with a flame ionization detector. For analysis, appropriate aliquots ($\sim 5 \mu\text{L}$) were taken from the individual FAME and MAGD samples. The SGG FAMEs were analyzed by GLC on a glass column (2m x 4mm) of 10% SP-2330 at 180°C and 215°C. The injector and detector temperatures were set at 45°C and 50°C, respectively, above the column temperature. SGG FAME peak identities were determined by comparison to retention times and separation factors (see Appendix Three) of standard FAMEs, which were chromatographed under similar conditions. Likewise, the SGG MAGDs were analyzed by GLC at a column temperature of 215°C and the peak identities were determined by comparison to retention times and separation factors of standard MAGDs, which were chromatographed under similar conditions.

2.6 Removal of Endogenous Cations from SGC and SGG

$\text{MeOH}:\text{CHCl}_3$ (1:1), was used to prepare solutions of SGC and SGG. Aqueous HCl (0.3 M) was added to the sulfatide solutions to give a $\text{MeOH}:\text{CHCl}_3:\text{HCl}$ ratio of 1:1:0.9. The mixtures were gently mixed, centrifuged (1700 rpm, 2 min), and the lower CHCl_3 phases were removed by pipetting. The upper aqueous phases were washed twice

with CHCl_3 to extract any remaining lipid, and the extracts were pooled together with the original extract fractions. The extracted lipid solutions were then neutralized with 0.01 N NaOH in MeOH, and the SGC- Na^+ and SGG- Na^+ salts were precipitated with acetone and dried *in vacuo* (Kates, 1986).

2.7 Preparation of Multilamellar Bilayers

Approximately 1 mg each of SGC- Na^+ , αGC , nGC, and mGC was hydrated (50% w/w) in 50 μM Tris-HCl in D_2O , pH 7.05, to form lipid multilamellar bilayers. To ensure complete dispersion, the lipid multilayers were heated and vortexed with the procedure being repeated 3-4 times. Multilayers of SGC were also generated in the presence of Ca^{2+} (SGC- Ca^{2+}) by including 1.5 M CaCl_2 in Tris-HCl in D_2O , pH 7.05. This yielded a 0.73:1 molar ratio of SGC to Ca^{2+} .

Approximately 1 mg of SGG- Na^+ was hydrated (50 wt%) in 50 μM Tris-HCl in $\text{D}_2\text{O}/\text{H}_2\text{O}$, pH 7.05, to form lipid multilamellar bilayers. Multilayers of SGG were also generated in the presence of Ca^{2+} , Mg^{2+} , and Sr^{2+} by including 1.9 M CaCl_2 in Tris-HCl in $\text{D}_2\text{O}/\text{H}_2\text{O}$, pH 7.05; 1.9 M MgCl_2 in Tris-HCl in $\text{D}_2\text{O}/\text{H}_2\text{O}$, pH 7.05; or 1.9 M SrCl_2 in Tris-HCl in $\text{D}_2\text{O}/\text{H}_2\text{O}$, pH 7.05, respectively. This yielded an approximate 1:1 molar ratio of SGG to $\text{Ca}^{2+}/\text{Mg}^{2+}/\text{Sr}^{2+}$.

Solid samples of SGC- Na^+ , SGC- Ca^{2+} , SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} were prepared by lyophilizing the lipid multilayers for ~ 24 h, followed by purging the samples with a N_2 stream for ~ 72 h. Solid samples of αGC , nGC, and mGC were exposed to a N_2 stream for 1 h to remove any water present.

2.8 Spectroscopic Analysis

For spectroscopic analysis, individual samples (~ 0.01 mg) were placed with powdered α -quartz (an internal pressure calibrant), in a well (0.37 mm diameter) of a stainless steel gasket (0.23 mm thickness), which was then mounted on a diamond anvil cell at room temperature (Wong et al., 1985).

A Digilab FTS-60 Fourier transform spectrophotometer or a Bomem-Michelson series 110 Fourier transform spectrophotometer were used to measure the infrared spectra at 28°C using a mercury cadmium telluride detector cooled by liquid N₂. The infrared beam was condensed onto the diamond anvil cell by a sodium chloride lens system. Prior to each measurement, the system was purged with a nitrogen stream for one minute to remove any water vapour or carbon dioxide present. Infrared spectra of all samples were measured as a function of pressure up to 17 kbar. For each spectrum, 512 interferograms were co-added at a spectral resolution 4 cm⁻¹.

A temperature-stabilized glowing ceramic was the source of high intensity infrared radiation and an internal He-Ne laser controlled the wavenumber precision. The detector was a liquid N₂-cooled mercury-cadmium-telluride (MCT) detector.

A translation program provided by the manufacturer was used to convert the raw spectra data into a DOS format. The data was then processed using software developed at the National Research Council, Ottawa (Moffatt et al., 1986). The 695 cm⁻¹ phonon band of the α -quartz was used to determine the hydrostatic pressure in the gasket of the diamond anvil (Wong et al., 1985). Application of pressure to the lipid multilayers reduces the mobility of the hydrocarbon chains, resulting in decreases in magnitude of

the intermolecular reorientational fluctuations and twisting and torsion motions and hence, 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 a correlation field splitting in the vibrational modes of the hydrocarbon chains (Auger et al., 1988, 1990). Therefore, when the barotropic behaviour of the vibrational modes of the hydrocarbon chains is monitored, the pressure required to induce this splitting can be used as a measure of how ordered or disordered the orientations of the hydrocarbon chains are; greater chain disorder requires more pressure to compress the chains into an ordered state (Refer to appendix 1 for complete explanation of HPFT-IR).

Fourier derivation and deconvolution techniques were applied in order to separate unresolvable infrared band contours. Spectral features originating from the vibrational modes of the sulfate group, the amide group (for SGC, α GC, nGC, and mGC), the ester group (for SGG), and the hydrocarbon chains were examined and height intensity ratios were determined from the vibrational modes originating from the hydrocarbon chains (for SGG). All data shown were reproducible. Figures shown are representative data.

CHAPTER THREE

RESULTS

3.1 Characterization of SGG, SGC and GC

Purity of the SGG, SGC, and GC samples was verified by TLC using the solvent system (CHCl_3 :MeOH:H₂O:conc. ammonia (65:25:3:1 v/v) (Kornblatt, 1979) and sugar staining with α -naphthol/H₂SO₄ (Figure 3.1) (Kates, 1986). All the samples with the exceptions of PC and PE, showed positive α -naphthol staining, verifying that they were glycolipids. Upon charring, the PC and PE bands were revealed with no extra bands (i.e., impurities) being observed among the glycolipid bands. The nGC sample gave a single band with its relative R_f value of 0.59, whereas that α GC sample chromatographed as doublets with relative R_f values of 0.52 and 0.48 (Table 3.1). These α GC doublets presumably represented two α GC molecular species with different fatty acyl chains. Two distinctive bands were observed for the mGC samples with relative R_f values of 0.56 and 0.51 (Table 3.1). Apparently, the fastest moving band of mGC represented nGC, and the slower moving band is α GC. The SGC sample chromatographed as doublets with relative R_f values of 0.37 and 0.39 (Table 3.1). These SGC doublets presumably represented the sulfatide molecular species with and without an α -hydroxyl group in the fatty acyl chain. The PE sample had a R_f value of 0.42 and PC chromatographed as doublets with relative R_f values of 0.29 and 0.27. The SGG sample chromatographed as a broad band with a relative R_f value of 0.43 (Table 3.1). The broad band was most likely due to the various molecular subclasses of SGG acyl and alkyl moieties.

Figure 3.1 - Thin-layer chromatogram illustrating co-chromatographed lipids: galactosylceramide with non-hydroxy fatty acid (nGC) (lane 1); galactosylceramide with α -hydroxy fatty acid (α GC) (lane 2); galactosylceramide with α -hydroxy fatty acid ($\sim 60\%$) and non-hydroxy fatty acid ($\sim 40\%$) (mGC) (lane 3); sulfogalactosylceramide with α -hydroxy fatty acid ($\sim 60\%$) and non-hydroxy fatty acid ($\sim 40\%$) (SGC) (lane 4); sulfogalactosylacylglycerol (SGG) (lane 5); and phosphatidylcholine (PC)/phosphatidylethanolamine (PE) (lane 6). *The lipids were separated using the solvent system $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}:\text{conc. ammonia}$ (65:25:3:1). The glycolipids were then visualized using α -naphthol spray followed by the general lipid spray 1:1 $\text{H}_2\text{SO}_4:\text{H}_2\text{O}$ and charring.*

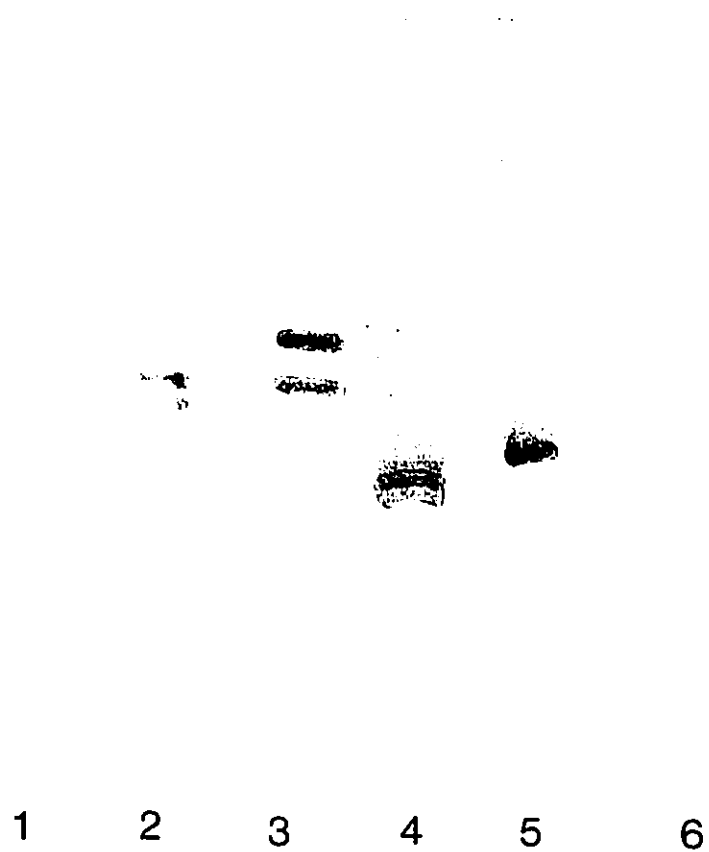


Figure 3.1

Table 3.1 R_f Values of nGC, α GC, mGC, SGC, and SGG separated by TLC using the solvent system CHCl_3 :MeOH:H₂O:conc. ammonia (65:25:3:1) and those based on results by Gadella et al., 1992.

Lipid	R_f^*	R_f^* (from Gadella et al., 1992) [†]
nGC	0.59	0.59
α GC	0.52	----
	0.48	0.50
mGC	0.56	0.55
	0.51	0.47
SGC	0.37	0.35
	0.39	0.38
SGG	0.43	0.43

* R_f = distance migrated by lipid band/distance migrated by solvent front

† Reference contains picture of their TLC. R_f values are based on my calculations.

The purity of SGG extracted from ram testes was also verified by comparing the infrared spectrum of SGG-Na⁺ (Figure 3.2B) with that described earlier for SGG extracted from boar testes (Figure 3.2A, from Ishizuka, Suzuki, and Yamakawa, 1973). Visual inspection of the two spectrums revealed no significant differences.

Figure 3.2 - Comparison of the infrared spectra of (A) boar testis sulfogalactosyl-acylalkylglycerol (SGG) in the frequency range 400-1800 cm^{-1} (obtained from Ishizuka et al., 1973) and (B) purified ram testis sulfogalactosyl-acylalkylglycerol (SGG) in the frequency range of 400-1800 cm^{-1} .

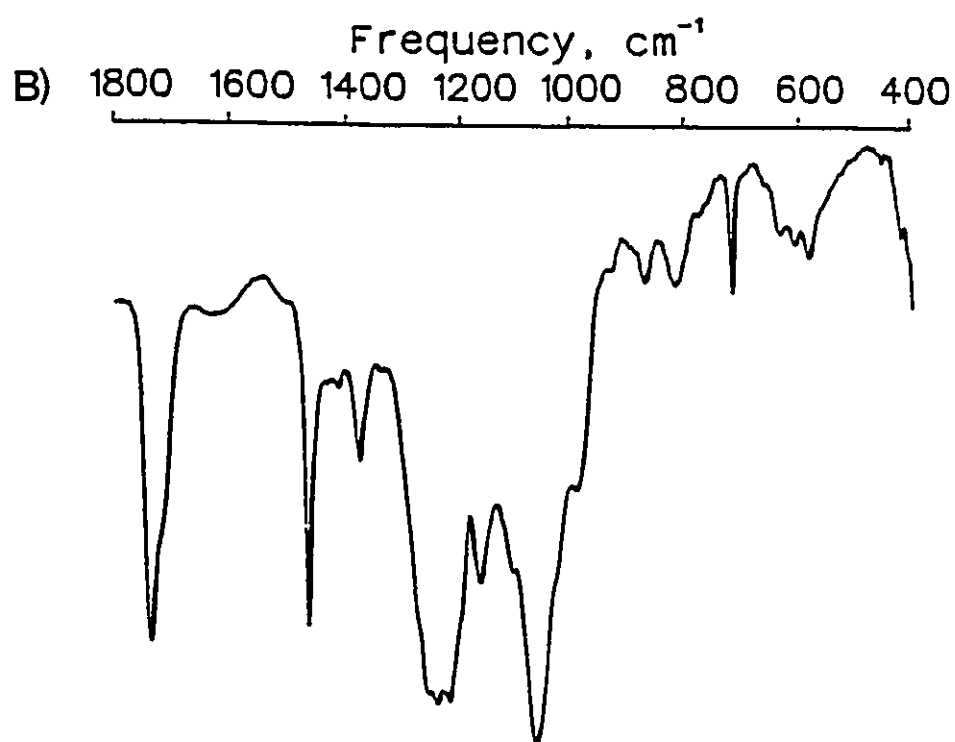
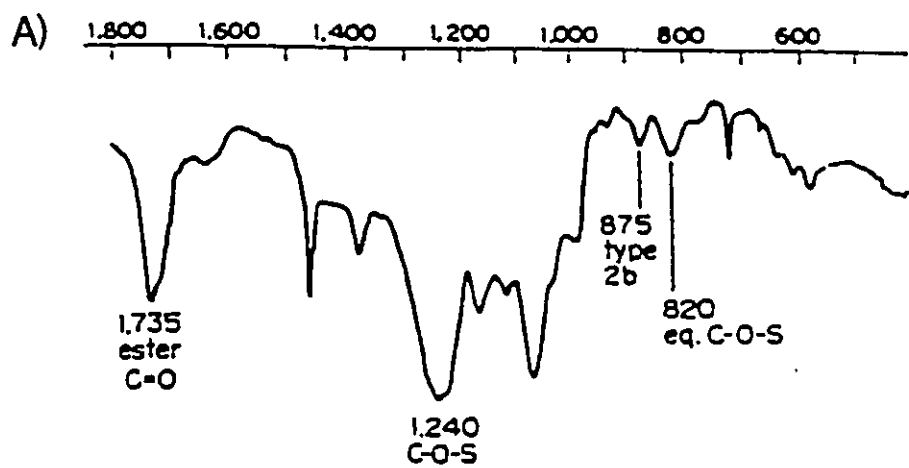


Figure 3.2

The acyl and alkyl moieties of SGG were characterized by methanolysis/acetylation of SGG followed by GLC analysis of the reaction products. The FAMES of SGG were derived from the acyl chain subclasses and identified by GLC analysis at 180°C and 215°C. The standard FAME chromatograms are shown in figures 3.3 (180°C) and 3.5 (215°C) and analyzed in tables 3.2 and 3.4, respectively. The SGG FAME chromatograms are shown in figures 3.4 (180°C) and 3.6 (215°C) and interpreted in tables 3.3 and 3.5, respectively.

The MAGDs of SGG were derived from the alkyl chain subclasses and characterized by GLC analysis at 215°C. SGG MAGD peaks were identified by comparison to the relative retention times of the standard MAGDS. The standard MAGD chromatogram is shown in figure 3.7 and analyzed in table 3.6. The SGG MAGD chromatogram is shown in figure 3.8 and interpreted in table 3.7. The overall results from the GLC characterization of the SGG acyl and alkyl moieties are summarized in table 3.8.

Characterization of the acyl subclass of SGG found that the major acyl group is ~95% palmitate (16:0), with small amounts of stearate (18:0, ~3%), myristate (14:0, <1%), and myristoleate (14:1, ~1%) (Table 3.8). The major alkyl group was found to be ~65% chimyl alcohol (16:0). Minor alkyl subclasses include tetradecanol (14:0, ~7%), pentadecanol (15:1, ~7%), pentadecanol (15:0, ~2%), and heptadecanol (17:0, ~1%) (Table 3.8)

Figure 3.3 - Gas-liquid chromatogram of standard fatty acid methyl esters (FAMES) separated at a column temperature of 180°C. Separated standard FAMES include C14:0 (peak 1), C16:0 (peak 2), C16:1 (peak 3), C18:0 (peak 4), and C18:1 (peak 5). *Data from chromatogram 3.3 are tabulated and analyzed in table 3.2.*

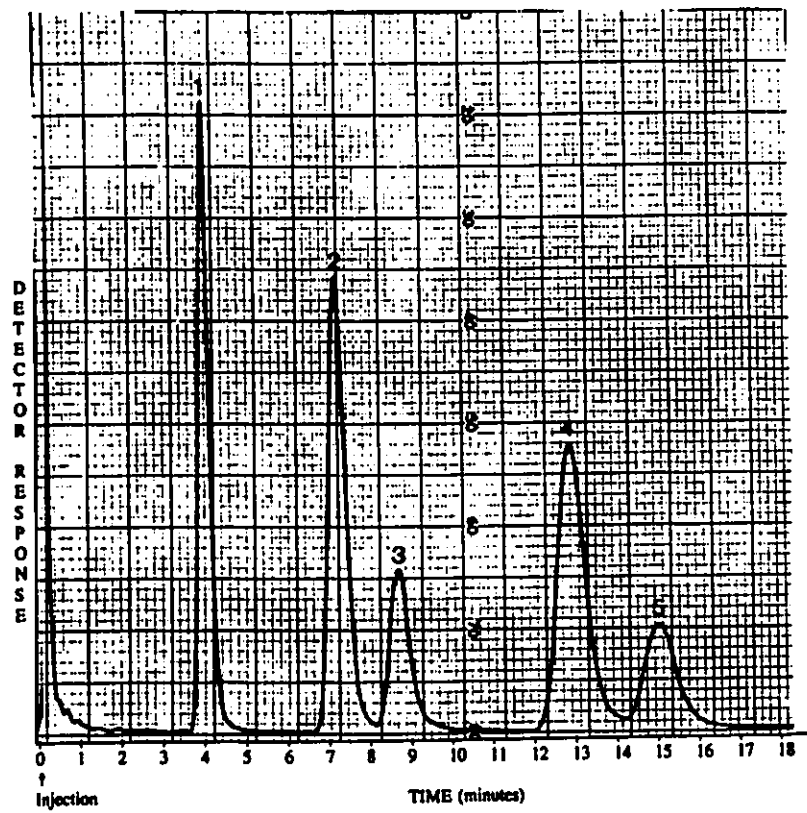


Figure 3.3

Table 3.2 Summary of standard FAME results from GLC analysis, 180°C (Figure 3.3)

Peak	Peak Distance* (mm)	Relative Retention Time (to peak #) [§]	Identity
1	40.0	1.0 (1)	14:0
2	71.5	1.79 = F ² (1)	16:0
3	87.0	1.22 = R (2)	16:1
4	129	1.80 = F ² (2)	18:0
5	150	1.16 = R (4)	18:1

Separation factors for SP 2330 at 180°C as determined from Figure 3.3:

2-CH₂ separation factor (F²) = 1.79

CH₂ separation factor (F) = 1.34

C=C separation factor (R) = (1.22 + 1.16)/2 = 1.19

§ Refer to Appendix Three for explanation of calculations.

* The distance of the peak from the origin is directly related to the retention time (Kates, 1986).

Figure 3.4 - Gas-liquid chromatogram of fatty acid methyl esters (FAMES) obtained from methanolysis reaction of sulfogalactosylacylglycerol (SGG). SGG FAMES were separated at a column temperature of 180°C. *Data from chromatogram 3.4 are tabulated and analyzed in table 3.3. Peak identities and separation factors were determined by comparison to data from figure 3.3 and table 3.2 (standard FAMES separated at 180°C).*

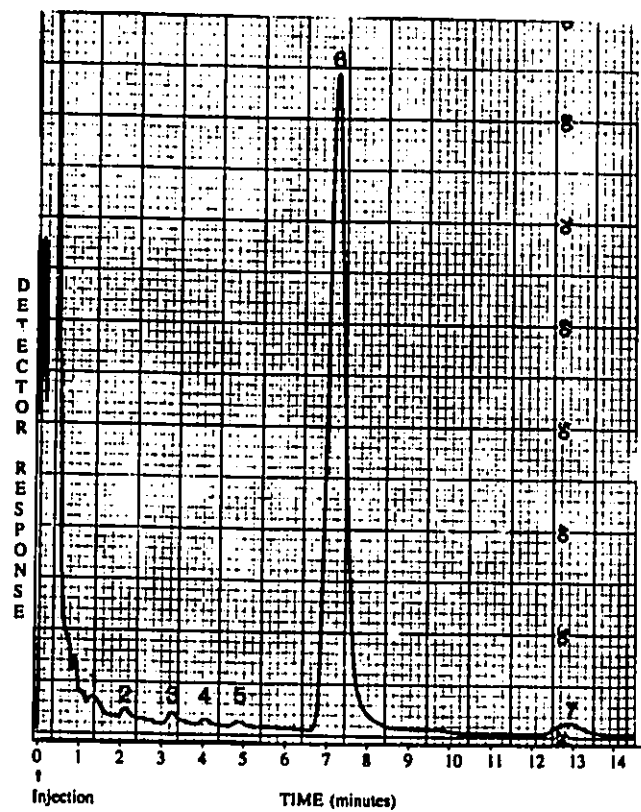


Figure 3.4

Table 3.3 Summary of SGG FAME results from GLC analysis, 180°C (Figure 3.4)

Peak	Peak Distance (mm)	Height of Peak (mm)	Area of Peak (t _r ·h)	% Area of Total Area [¶]	Relative Retention time (to peak #) [§]	Identity*
1	13.5	2.0	27	0.22	0.34 (4)	ND
2	20.5	2.0	41	0.33	0.51 (4)	ND
3	32.0	3.0	96	0.78	0.80 (4)	ND
4	40.0	2.0	80	0.65	1.00 (4)	14:0
5	49.0	2.0	98	0.80	1.22=R (4)	14:1
6	71.5	160	11,440	93.5	1.79=F ² (4)	16:0
7	128	3.0	384	3.1	3.20=F ⁴ (4)	18:0

* ND = Not determined

¶ Total area = 12,241 mm²

§ Refer to Appendix Three for explanation of calculations.

Figure 3.5 - Gas-liquid chromatogram of standard fatty acid methyl esters (FAMES) separated at a column temperature of 215°C. Separated standard FAMES include C14:0 (peak 1), C16:0 (peak 2), C16:1 (peak 3), C18:0 (peak 4), and C18:1 (peak 5). *Data from chromatogram 3.5 are tabulated and analyzed in table 3.4.*

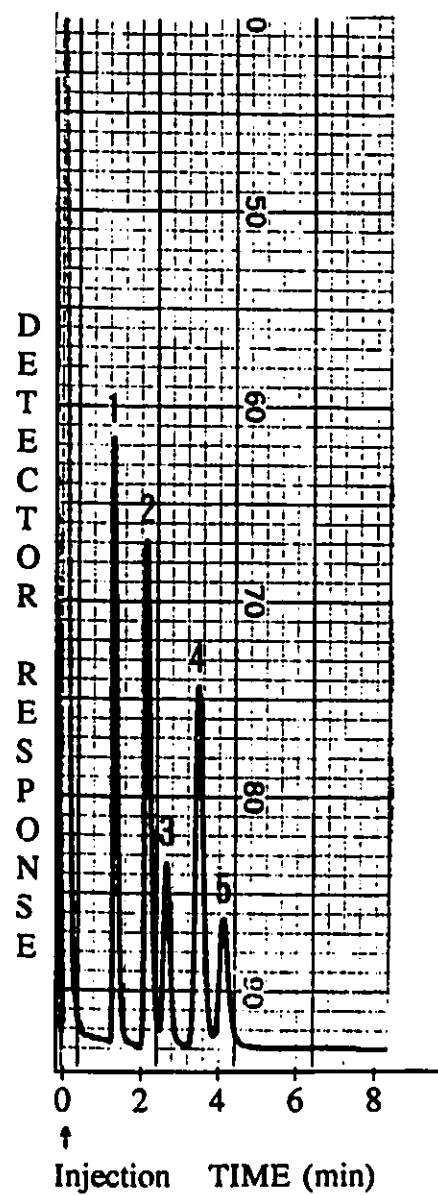


Figure 3.5

Table 3.4 Summary of standard FAME results from GLC analysis, 215°C (Figure 3.5)

Peak	Peak Distance (mm)	Relative Retention Time (to peak #) [§]	Identity
1	7.0	0.61 = F ² (2)	14:0
2	11.5	1.00 (2)	16:0
3	13.5	1.17 = R (2)	16:1
4	17.5	1.52 = F ² (2)	18:0
5	21.0	1.20 = R (4)	18:1

Separation factors for SP 2330 at 215°C as determined from figure 3.5:

2-CH₂ separation factor (F²) = 1.57

CH₂ separation factor (F) = 1.26

C=C separation factor (R) = 1.19

§ Refer to Appendix Three for explanation of calculations.

Figure 3.6 - Gas-liquid chromatogram of fatty acid methyl esters (FAMES) obtained from methanolysis reaction of sulfogalactosylalkylglycerol (SGG). SGG FAMES were separated at a column temperature of 215°C. *Data from chromatogram 3.6 are tabulated and analyzed in table 3.5. Peak identities and separation factors were determined by comparison to data from figure 3.5 and table 3.4 (standard FAMES separated at 215°C).*

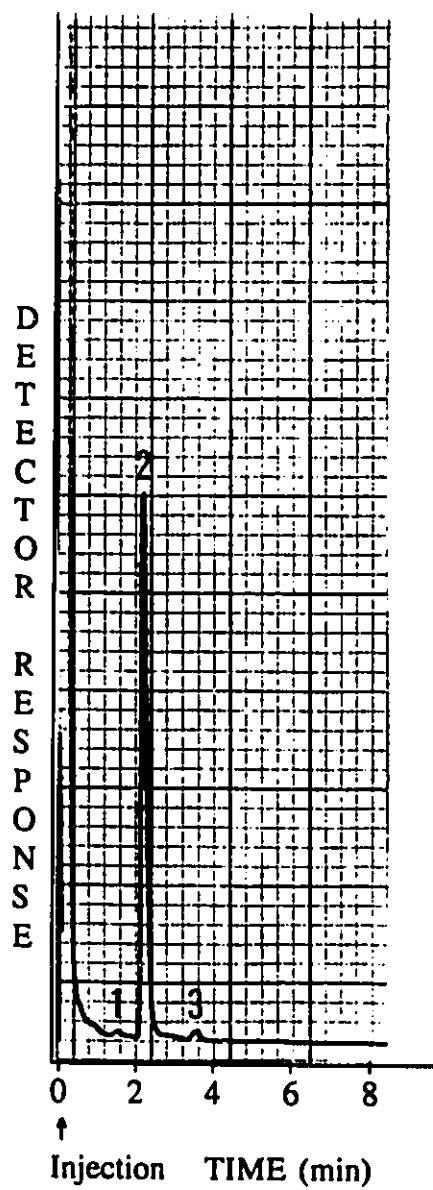


Figure 3.6

Table 3.5 Summary of SGG FAME results from GLC analysis, 215°C (Figure 3.6)

Peak	Peak Distance (mm)	Height of Peak (mm)	Area of Peak ($t_R h_n$)	% Area of Peak [¶]	Relative Retention Time (to peak #) [§]	Identity
1	7.5	1.5	11	1.3	0.65 = F ² (2)	14:0
2	11.5	70.0	805	95.5	1.00 (2)	16:0
3	17.5	1.5	26	3.1	1.52 = F ² (2)	18:0

¶ Total Area = 842 mm²

§ Refer to Appendix Three for explanation of calculations.

Figure 3.7 - Gas-liquid chromatogram of standard monoalkyl glycerol diacetates (MAGDs) obtained from methanolysis/acetylation reaction of 1-O-hexadecyl-sn-glycerol and 1-O-octadecyl-sn glycerol. Standard MAGDs were separated at a column temperature of 215°C. Separated standard MAGDs include C16:0 (peak 1), C18:0 (peak 2), and C20:0 (peak 3). *Data from chromatogram 3.7 were tabulated and analyzed in table 3.6.*

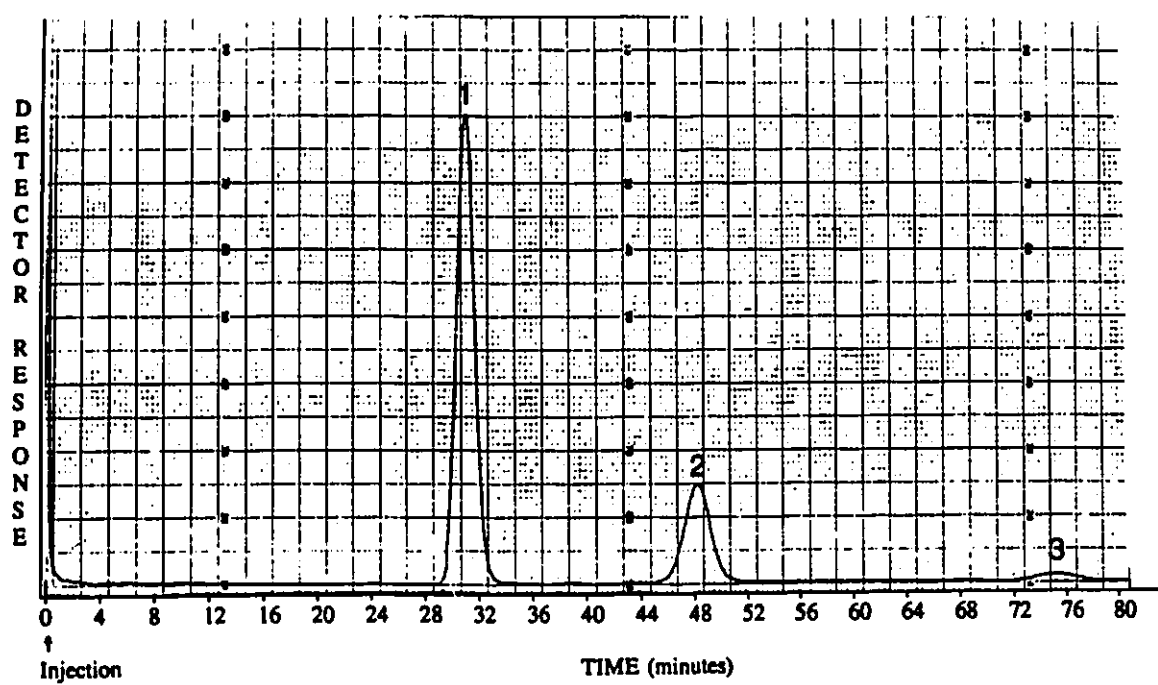


Figure 3.7

Table 3.6 Summary of standard MAGD results from GLC analysis, 215°C (Figure 3.7)

Peak	Peak Distance (mm)	Relative Retention Time (to peak #) [§]	Identity
1	154.5	1.00 (1)	16:0
2	239.5	1.55 = F ² (1)	18:0
3	374.5	1.56 = F ² (2)	20:0

Separation factors for SP 2330 at 215° as determined from Figure 3.7:

2-CH₂ separation factor (F²) = 1.56

CH₂ separation factor (F) = 1.25

§ Refer to Appendix Three for explanation of calculations.

Figure 3.8 - Gas-liquid chromatogram of monoalkyl glycerol diacetates (MAGDs) obtained from methanolysis/acetylation reaction of sulfogalactosyl-acylalkylglycerol (SGG). SGG MAGDs were separated at a column temperature of 215°C. *Data from chromatogram 3.8 are tabulated and analyzed in table 3.7. Peak identities and separation factors were determined by comparison to data from figure 3.7 and table 3.6 (standard MAGDs separated at 215°C).*

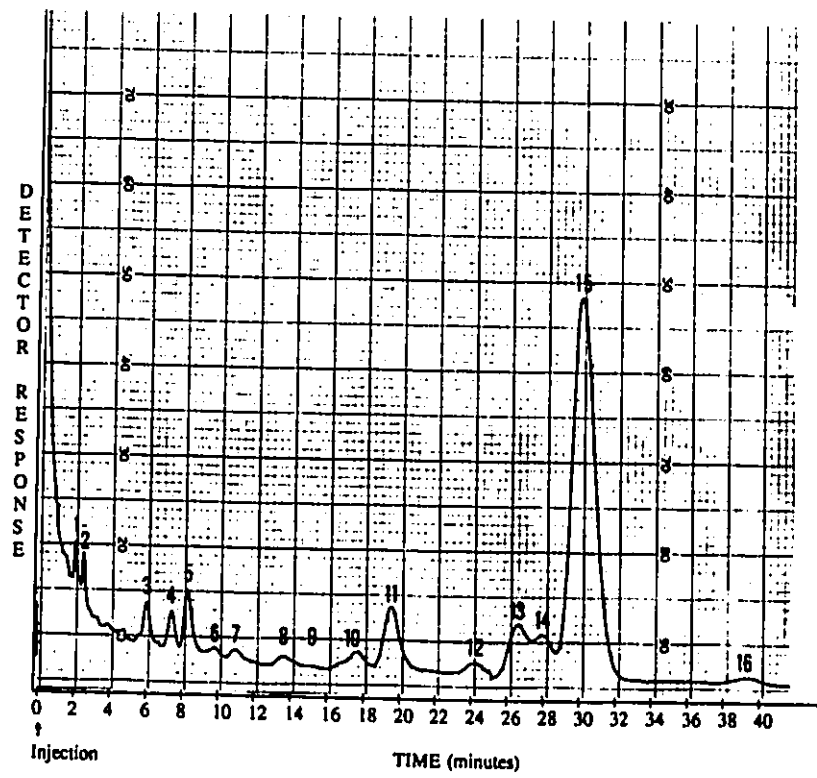


Figure 3.8

Table 3.7 Summary of SGG MAGD results from GLC analysis, 215°C (Figure 3.8)

Peak	Peak Distance (mm)	Height of Peak (mm)	Area of Peak ($t_n \cdot h_n$)	% Area of Peak [†]	Relative Retention Time (to peak #) [§]	Identity*
1	11.0	11.0	121	0.48	-----	ND
2	12.5	13.0	162	0.65	$0.40 = F^{-4}$ (6)	5:0
3	31.0	12.0	372	1.5	$0.63 = F^{-2}$ (6)	9:0
4	37.5	10.0	375	1.5	$1.21 = R$ (3)	9:1
5	42.0	16.5	693	2.8	-----	ND
6	49.5	2.0	99	0.39	$0.65 = F^{-2}$ (9)	11:0
7	55.5	3.0	167	0.66	-----	ND
8	69.0	3.0	207	0.82	$1.40 = R^2$ (6)	11:2
9	76.5	1.0	77	0.30	$0.78 = F^{-1}$ (11)	13:0
10	89.0	5.0	445	1.8	$1.16 = R$ (9)	13:1
11	98.5	18.0	1773	7.1	$0.65 = F^{-2}$ (15)	14:0
12	122.0	3.5	427	1.7	$0.81 = F^{-1}$ (15)	15:0
13	132.0	15.0	1980	7.9	-----	ND
14	140.0	12.0	1686	6.7	$1.15 = R$ (12)	15:1
15	151.5	107.0	16211	64.6	1.00 (15)	16:0
16	198.5	1.5	298	1.2	$1.31 = F$ (15)	17:0

* ND = Not determined

† Total area = 25,092 mm²

§ Refer to Appendix Three for explanation of calculations.

Table 3.8 Overall Molecular Subclass Composition of SGG

Molecular Subclass	% Composition of Major Fatty Acids and Glyceryl Ethers ^{*†}							
	14:0	14:1	15:0	15:1	16:0	16:1	17:0	18:0
Acyl	0.65	1.1	----	----	95	----	----	3.1
Alkyl [*]	7.1	----	1.7	6.7	65	----	1.2	----

* There was a major MAGD peak (Figure 3.8, peak 13, 7.9%) whose identity could not be calculated.

† There were several other molecular subclasses revealed in the GLC analysis, however their individual % composition was, in general, less than 1-2%.

3.2 Infrared Analysis of SGC and GC

The samples of SGC and mGC used in this study are both mixtures of α -hydroxy ($\sim 60\%$) and non-hydroxy ($\sim 40\%$) fatty acids. The frequencies of the SGC sulfate absorption bands were verified by comparison to the mGC spectra, the nonsulfated parental compound of SGC. To determine the structural and dynamic effects of fatty acid α -hydroxylation, the spectra of nGC and α GC were also studied.

To verify Ca^{2+} binding to the SGC sulfate moiety, two sulfate absorption modes were analyzed: the asymmetric S=O stretching mode ($\nu_{\text{as}}\text{S}=\text{O}$) and the C-O stretching mode ($\nu\text{C}-\text{O}$) of the ester-linked sulfate group. Due to overlapping C-OH stretching bands from the sugar head group, the S=O symmetrical stretching mode ($1040\text{-}1080\text{ cm}^{-1}$) could not be used for the analysis (Kates, 1986). Also, the S-O stretching mode ($780\text{-}860\text{ cm}^{-1}$) could not be properly analyzed due to interfering quartz absorption modes (Kates, 1986; Wong et al., 1985). The O-H and N-H stretching region was also analyzed to verify that no interaction was occurring between the Ca^{2+} and the sugar O-H groups and amide N-H group.

Any changes to the hydrogen bonding in the interfacial region, as a result of Ca^{2+} binding to the sulfate moiety in the polar head region of SGC, would be reflected in the SGC amide I absorption mode. Also, α -hydroxylation of the fatty acid would be expected to affect the hydrogen bonding in the interfacial region. Any hydrogen bonding effects of α -hydroxylation on the interfacial region would be revealed by comparison of the absorption mode of α GC, nGC, and mGC.

The binding of Ca^{2+} to the sulfate moiety in the polar head region of SGC and

the α -hydroxylation of the fatty acid would be expected to not only affect the hydrogen bonding in the interfacial region but also affect the dynamics of the hydrocarbon chains. The conformational and orientational order/disorder of the hydrocarbon chains ultimately determine the "dynamics" of the chains and bilayer. The δCH_2 scissoring and γCH_2 rocking modes are sensitive to any changes in the order/disorder dynamics of the hydrocarbon chains (Stein and Sutherland, 1953, 1954). The correlation field splitting pressure of the δCH_2 mode can be used to determine the effect of Ca^{2+} -SGC binding on the dynamics of the SGC hydrocarbon chains. Moreover, any changes to the bilayer midplane are reflected in the CH_3 bending mode of the hydrocarbon chains.

3.2.1 Calcium Binding to the Negatively-Charged Sulfate Moiety

The $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching mode absorbs in the frequency range of 1200 to 1265 cm^{-1} (Kates, 1986). Solid samples of SGC- Na^+ , SGC- Ca^{2+} , and mGC were used for the Ca^{2+} binding study due to the presence of interfering D_2O bands. The spectra of solid SGC- Na^+ , SGC- Ca^{2+} , and mGC in the $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching region at ambient pressure are shown in figure 3.9. In this frequency range, the spectrum of mGC showed no major absorption as opposed to the spectra of SGC- Na^+ and SGC- Ca^{2+} , which both showed a major peak absorption at 1220 cm^{-1} . Upon addition of Ca^{2+} , the SGC $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching mode decreased in frequency from 1219 to 1215 cm^{-1} .

Figure 3.9 - The infrared spectra of solid SGC- Na^+ (—), SGC- Ca^{2+} (----), and mGC (—·—) in the S=O asymmetric stretching region (1200-1240 cm^{-1}) at ambient pressure.

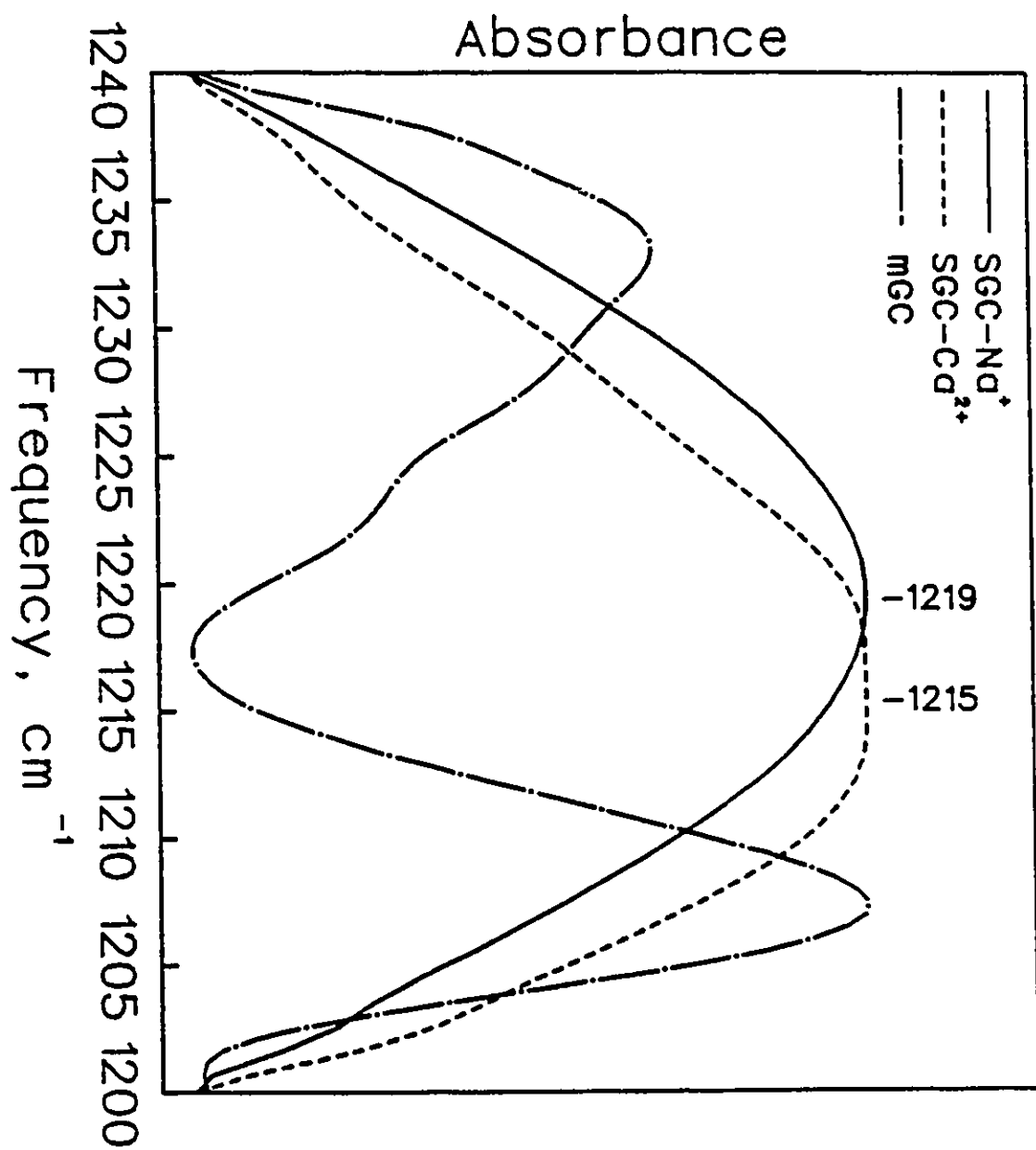


Figure 3.9

The ν C-O stretching mode of the sulfate group absorbs in the frequency range of 950 to 1020 cm^{-1} (Kates, 1986). The infrared spectra of hydrated SGC- Na^+ , SGC- Ca^{2+} , and mGC are shown in this frequency region at ambient pressure in figure 3.10. The spectrum of mGC lacked a major absorption in this range, whereas both SGC- Na^+ and SGC- Ca^{2+} had a major peak absorption at 990 cm^{-1} . Upon addition of Ca^{2+} , the SGC ν C-O stretching mode decreased slightly in frequency from 992 to 991 cm^{-1} .

The O-H and N-H stretching modes absorb in the frequency range of 3100 to 3700 cm^{-1} (Kates, 1986). Figure 3.11 shows the infrared spectra of solid SGC- Na^+ and SGC- Ca^{2+} in this frequency region at ambient pressure. The maximum absorption by each sample is well below 1.0 in this frequency range. Upon addition of Ca^{2+} to SGC, the overall bandshape changed and the broad overlapping O-H and N-H stretching modes increased slightly in peak absorption frequency.

Figure 3.10 - The infrared spectra of hydrated SGC- Na^+ (—), SGC- Ca^{2+} (----), and mGC (—·—) in the C-O stretching region (950-1020 cm^{-1}) at ambient pressure.

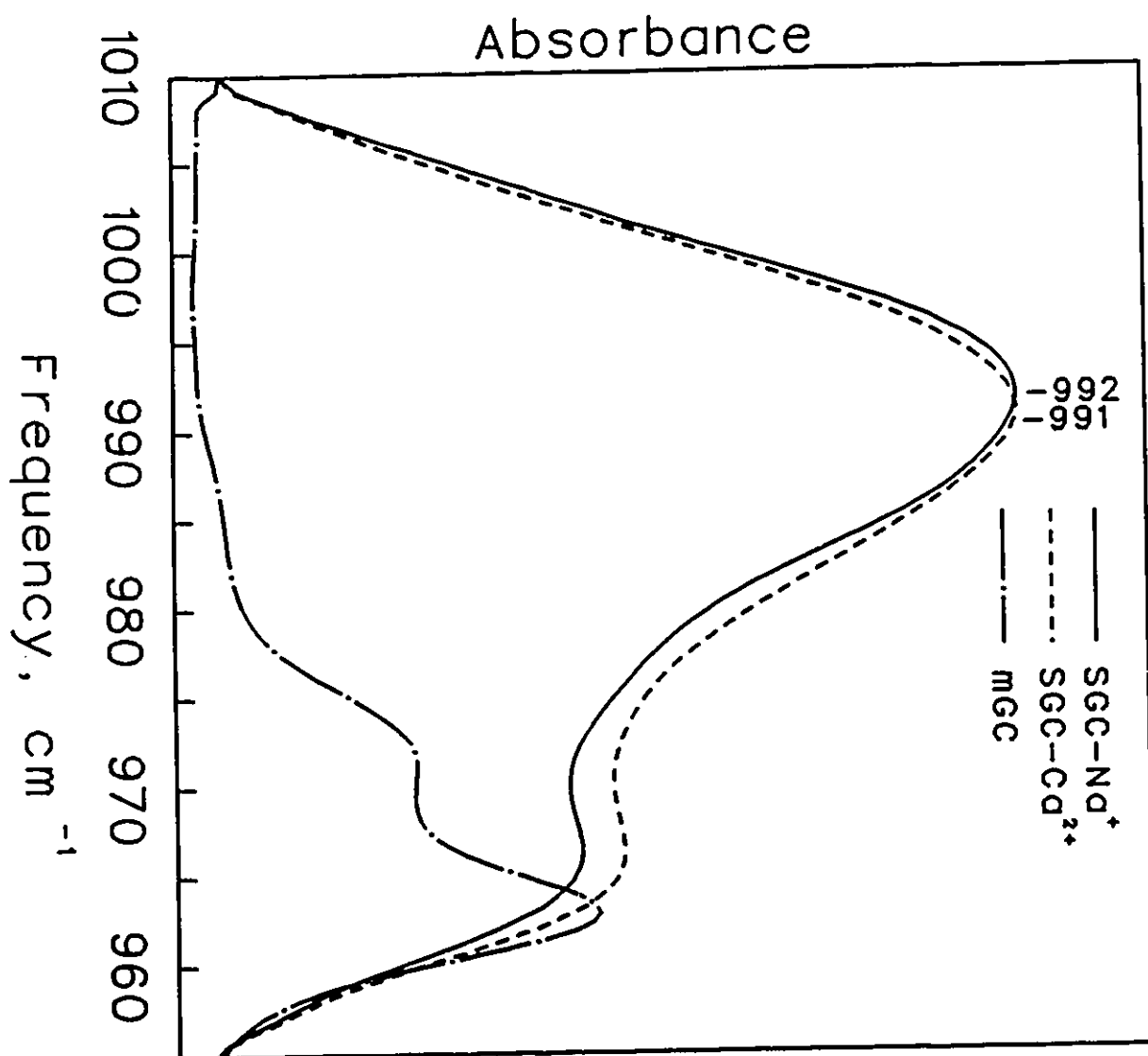


Figure 3.10

Figure 3.11 - The infrared spectra of solid SGC- Na^+ (—) and SGC- Ca^{2+} (----) in the O-H and N-H stretching region ($3100\text{-}3700\text{ cm}^{-1}$) at ambient pressure.

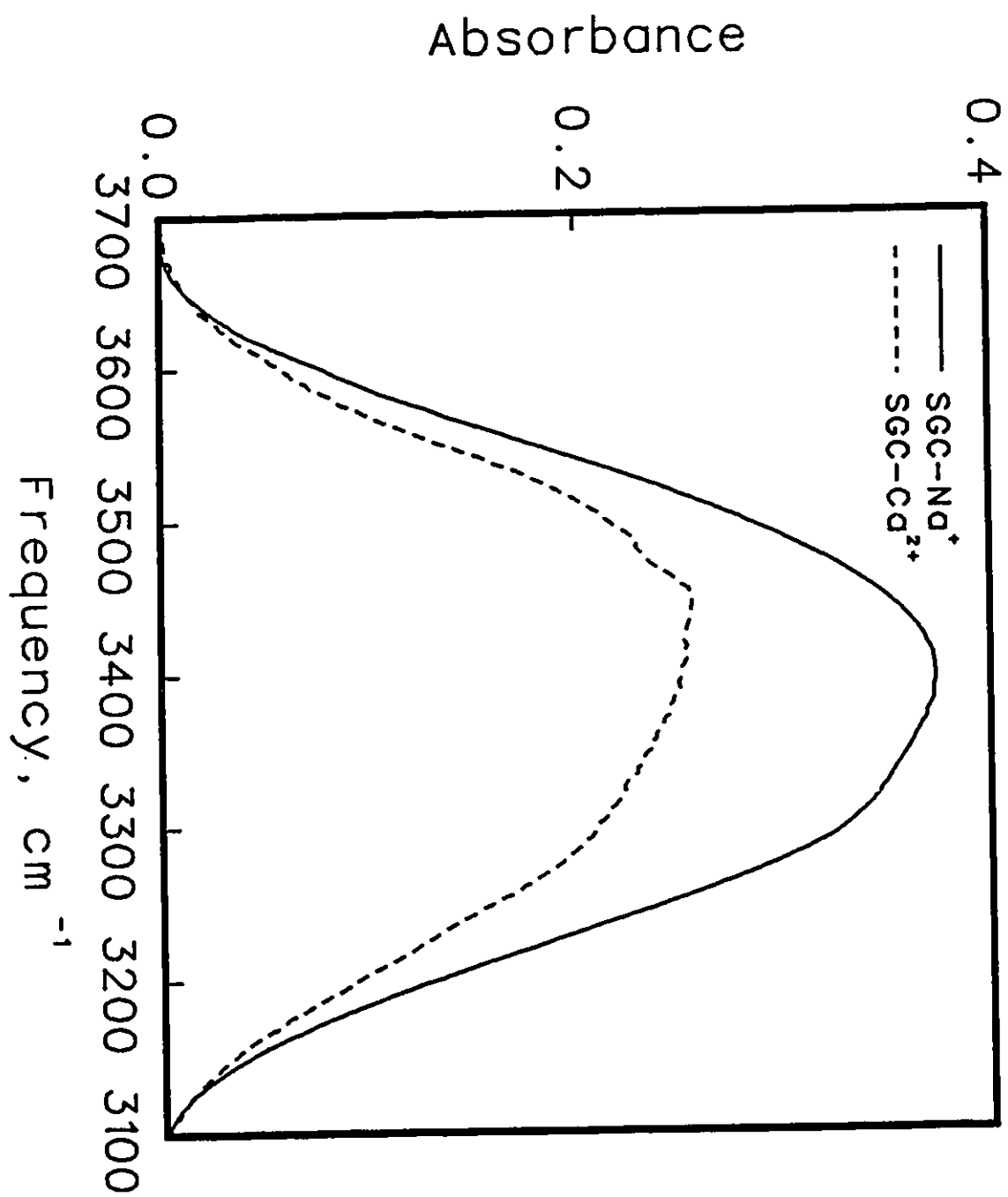


Figure 3.11

3.2.2 *The Interfacial Region (Amide Group)*

The amide I C=O stretching mode ranges from 1600 to 1700 cm^{-1} (Pimentel and McClellan, 1960). Figure 3.12 shows the infrared spectra of solid α GC, nGC, and mGC in this frequency range at ambient pressure. The amide I mode of nGC absorbed at the frequency of 1646 cm^{-1} , whereas for α GC, the absorption mode occurred at 1627 cm^{-1} , a significantly lower frequency. The spectrum of mGC revealed two components in this absorption mode: a high-frequency component at 1647 cm^{-1} and a low-frequency and low intensity component at 1627 cm^{-1} .

Analysis of the amide I C=O stretching mode of hydrated SGC- Na^+ and SGC- Ca^{2+} revealed a broad absorption from 1600 to 1670 cm^{-1} . The broad band was comprised of two components: a high-frequency component and a low-frequency component (Figure 3.13A). The third-order Fourier-transform derivative spectra of the same region, using a breakpoint of 0.2 in the Fourier domain, is shown in figure 3.13B. The derivative spectra reveal the individual components that comprised the broad bands in the original spectra in figure 3.13A. Upon Ca^{2+} addition to SGC, the high frequency component of the amide I C=O stretching mode decreased in intensity and increased slightly in frequency from 1647 to 1651 cm^{-1} . The low-frequency component of the SGC amide I band changed neither in intensity nor frequency (1619 cm^{-1}) upon Ca^{2+} addition.

Figure 3.12 - The infrared spectra of solid mGC (—), nGC (— · —), and α GC (----) in the amide I C=O stretching region (1600-1700 cm^{-1}) at ambient pressure.

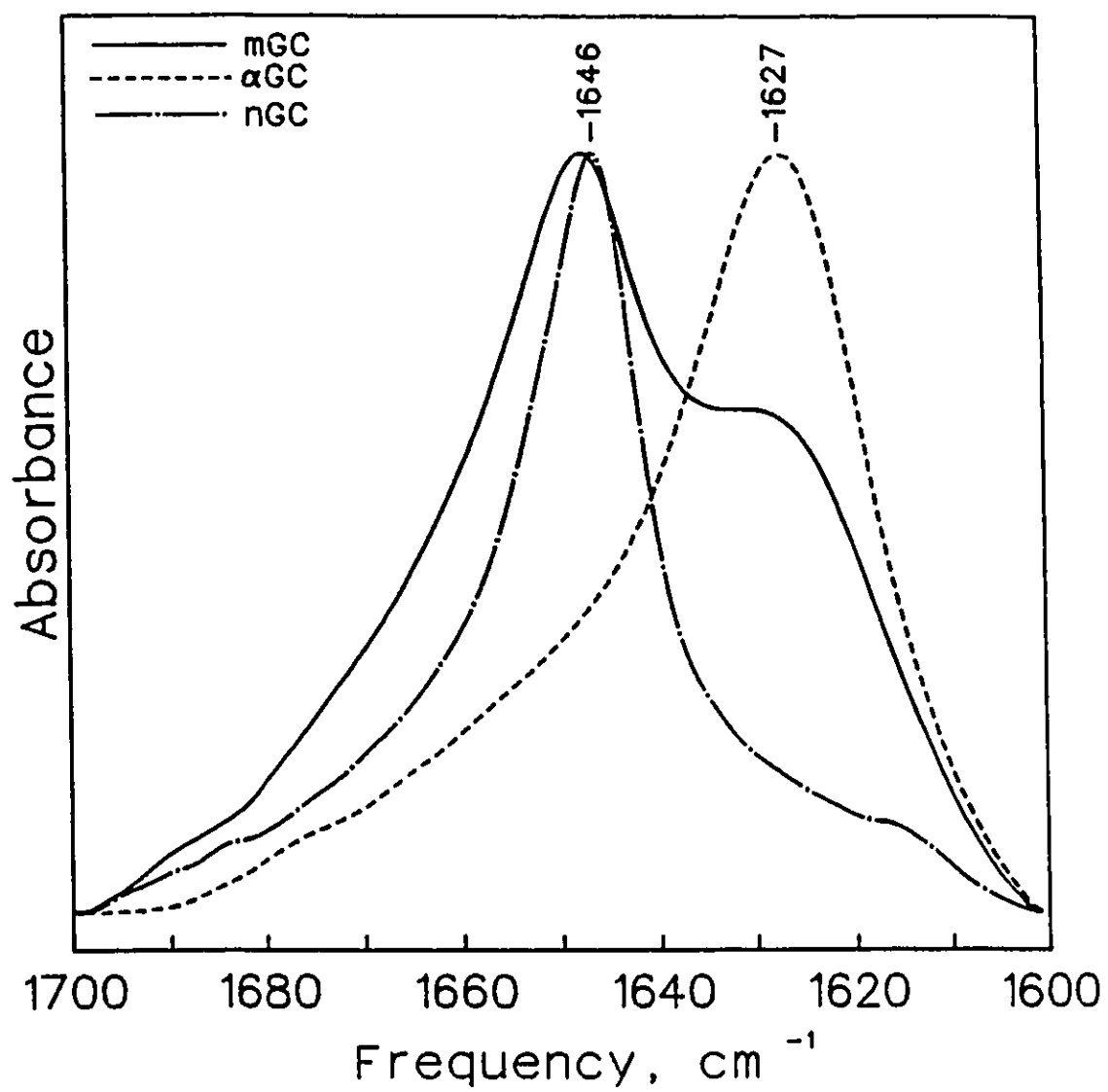


Figure 3.12

Figure 3.13 - (A) The infrared spectra of hydrated SGC- Na^+ (—) and SGC- Ca^{2+} (----) in the amide I C=O stretching region (1600-1700 cm^{-1}) at ambient pressure.
(B) The third-order Fourier-derivative spectra of hydrated SGC- Na^+ (—) and SGC- Ca^{2+} (----) in the amide I C=O stretching region, using a breakpoint of 0.2 in the Fourier domain.
Note: Spectra are baseline corrected.

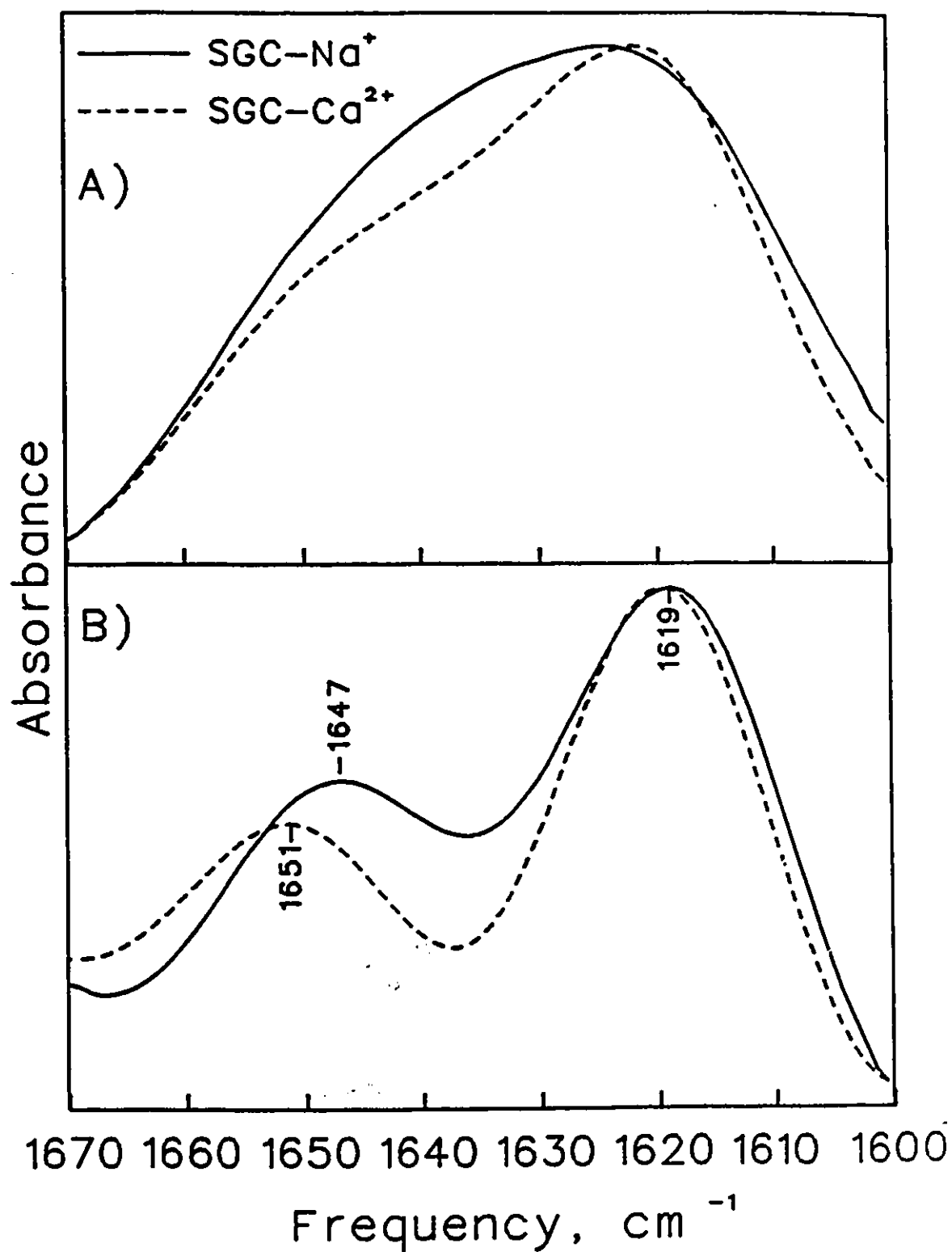


Figure 3.13

3.2.3 The Hydrocarbon Chain Structure

The δCH_2 scissoring mode absorbs in the region of 1430 to 1500 cm^{-1} (Kates, 1986). Figure 3.14 presents the stacked contour plots of the infrared spectra of hydrated nGC (A) and α GC (B) in the δCH_2 absorption region. At ambient pressure, both nGC and α GC spectra display an intense, narrow peak absorption at 1467 cm^{-1} . However, both spectra differ in their barotropic behaviour; increasing pressure induced a correlation field splitting in the δCH_2 mode of α GC at pressure values above 5.4 kbar (figure 3.14B). In contrast, the correlation field splitting of the δCH_2 mode is barely noticeable in the nGC spectra (figure 3.14A). However, some changes did occur; the nGC δCH_2 band increased in frequency and the band width became narrow with increasing pressure.

Figure 3.15 presents the stacked contour plots in the region of the δCH_2 mode of the infrared spectra of mGC. The barotropic behaviour of the absorption mode is similar to that of α GC. At ambient pressure, the mGC spectrum displayed an intense narrow peak absorption at 1467 cm^{-1} . However, the correlation field splitting of the δCH_2 mode of mGC was induced at a much lower pressure of 2.4 kbar.

Figure 3.14 - Stacked contour plots of the infrared spectra of the CH₂ scissoring mode (1430-1500 cm⁻¹) of (A) hydrated nGC and (B) hydrated α GC. The numbers on the right of the plots are pressure values in kbar.

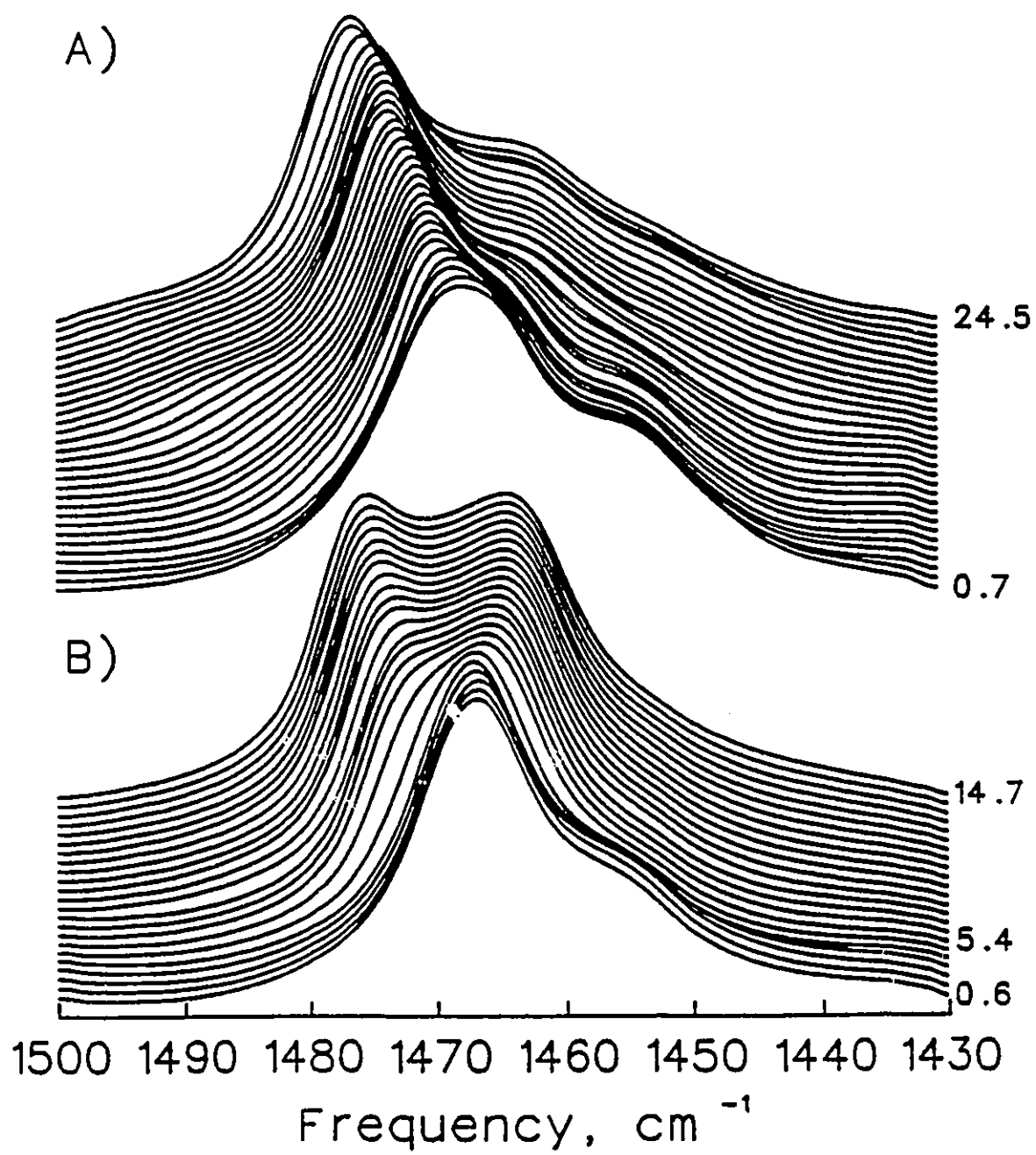


Figure 3.14

Figure 3.15 - Stacked contour plots of the infrared spectra of the CH₂ scissoring mode (1430-1500 cm⁻¹) of hydrated mGC. The numbers on the right of the plots are pressure values in kbar.

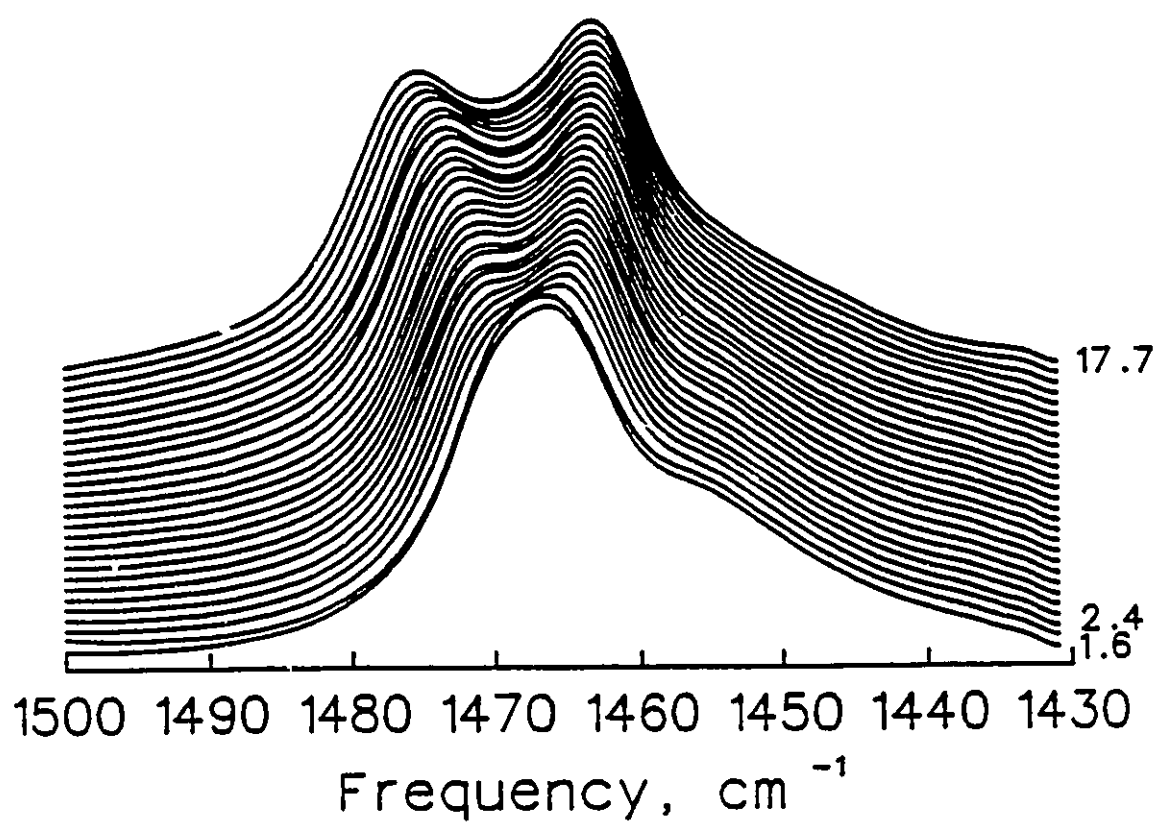


Figure 3.15

Stacked contour plots of the infrared spectra of hydrated SGC- Na^+ (A) and SGC- Ca^{2+} (B) in the δCH_2 scissoring region are shown in figure 3.16. Comparison of the spectra revealed that, with the exception of the splitting pressure, the barotropic behaviour of the δCH_2 mode was very similar in SGC- Na^+ and SGC- Ca^{2+} . At ambient pressure, both spectra had a narrow peak absorption at 1467 cm^{-1} . However, as illustrated by a plot of the pressure dependence of the δCH_2 mode frequencies (Figure 3.17), a correlation field splitting of the SGC- Ca^{2+} δCH_2 mode was induced at pressure values above 6.3 kbar, whereas the SGC- Na^+ δCH_2 mode split at pressures above 4.3 kbar. The pressure dependence of the mGC δCH_2 mode, whose correlation field splitting pressure is 2.4 kbar, was included in this figure to demonstrate the effect the presence or absence of the sulfate group has on the hydrocarbon chain dynamics.

The CH_3 symmetrical bending mode of the hydrocarbon chains absorbs in the region of 1355 to 1395 cm^{-1} (Bellamy, 1975). Figure 3.18 shows a plot of the pressure dependence of this mode for SGC- Ca^{2+} in the frequency range of 1377 to 1381 cm^{-1} . Both samples show similar barotropic behaviours, an increase in peak absorption frequency with increasing pressure. However, the peak absorption frequencies of SGC- Ca^{2+} (1377.5 cm^{-1} at ambient pressure) are consistently lower than those of SGC- Na^+ (1378.5 cm^{-1} at ambient pressure), with a difference of $\sim 1\text{ cm}^{-1}$ throughout the pressure range studied.

Figure 3.16 - Stacked contour plots of the infrared spectra of the CH₂ scissoring mode (1430-1500 cm⁻¹) of (A) hydrated SGC-Na⁺ and (B) hydrated SGC-Ca²⁺. The numbers on the right of the plots are pressure values in kbar.

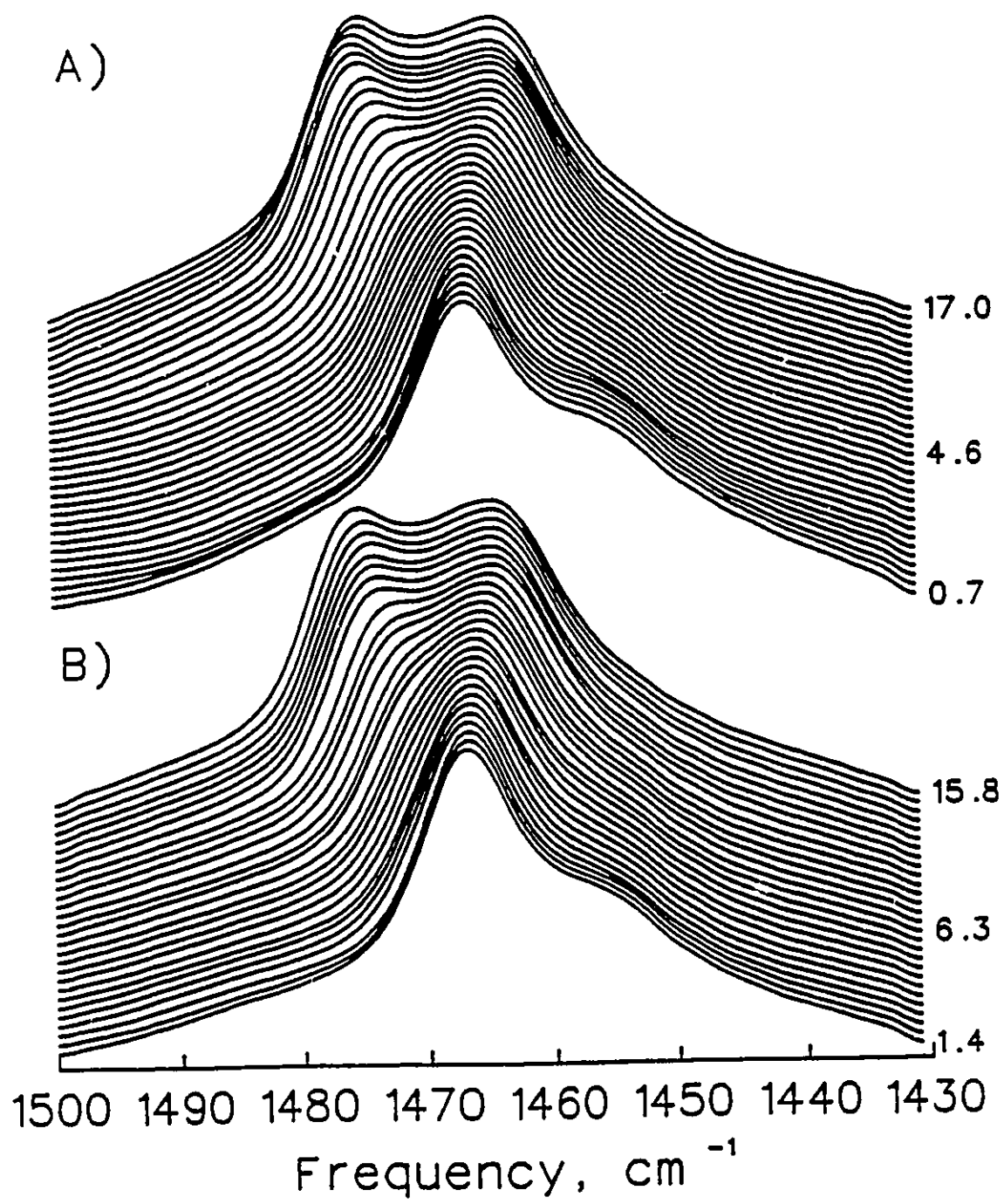


Figure 3.16

Figure 3.17 - Pressure dependence of the frequencies of the CH₂ scissoring mode (1460-1480 cm⁻¹) for hydrated SGC-Na⁺ (+), hydrated SGC-Ca²⁺ (○), and hydrated mGC (●). The numbers in parentheses are the splitting pressures in kbar. *Frequencies were determined from third-order derivative spectra, calculated using a breakpoint of 0.95 in the Fourier domain.*

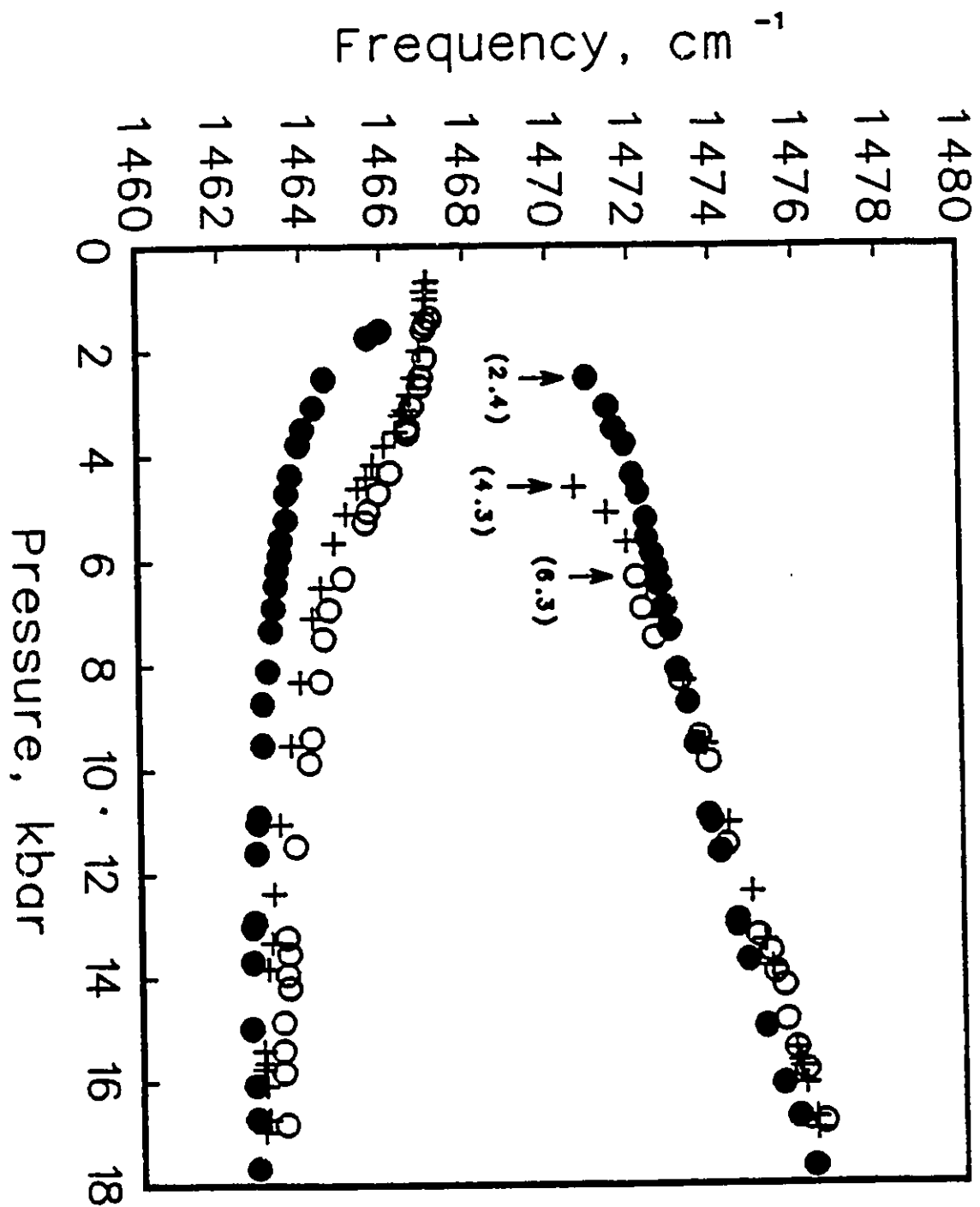


Figure 3.17

Figure 3.18 - Pressure dependence of the frequencies of the CH₃ bending mode (1377-1381 cm⁻¹) for hydrated SGC-Na⁺ (+) and hydrated SGC-Ca²⁺ (○). *Frequencies were determined from third-order derivative spectra, calculated using a breakpoint of 0.3 in the Fourier domain.*

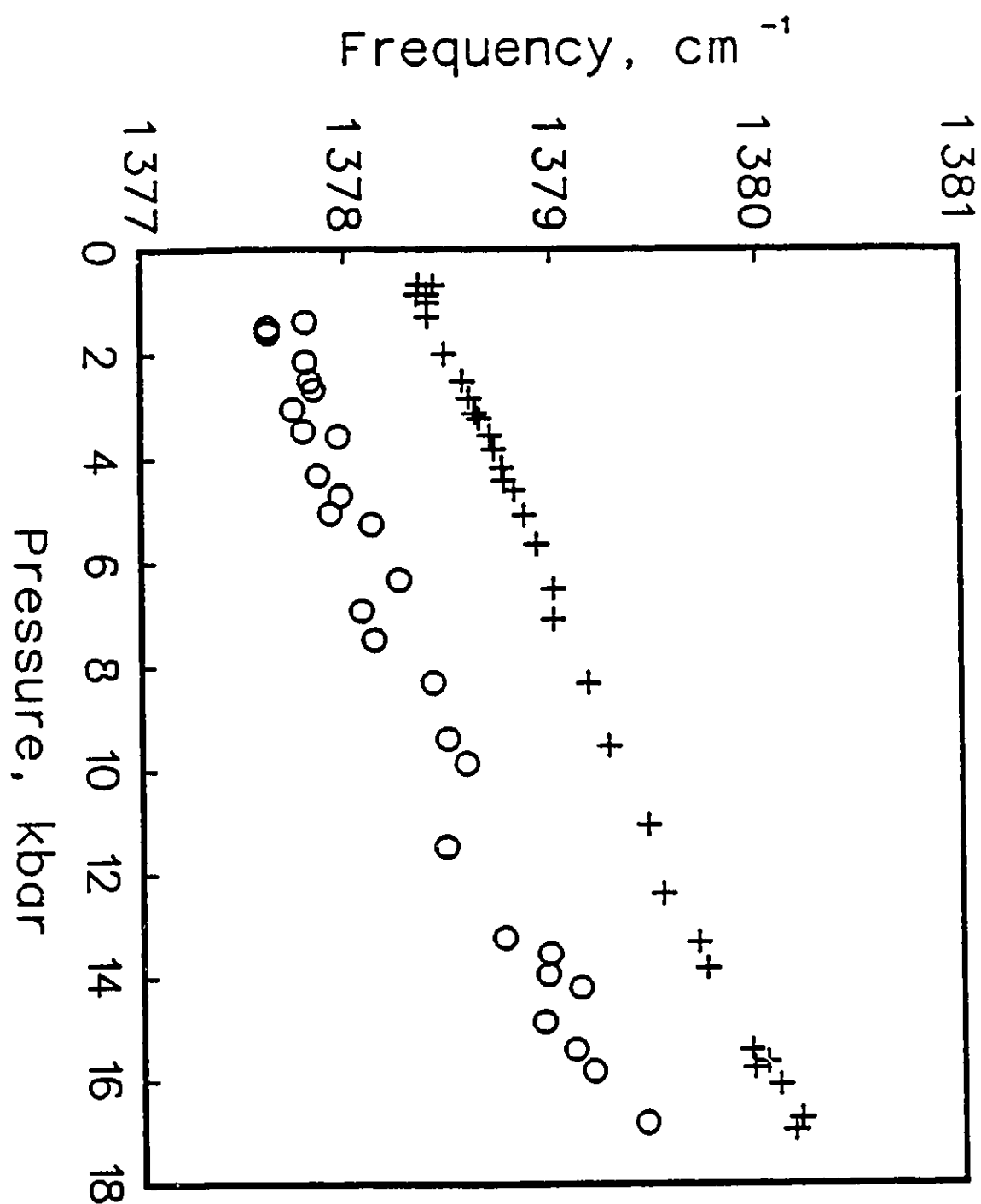


Figure 3.18

3.3 Infrared Analysis of SGG

Given the similar polar head groups of SGC and SGG, the SGG sulfate absorption bands were assumed to be located in the same frequency regions as those of SGC. To verify the divalent cation interaction with the SGG sulfate moiety, several sulfate absorption modes were analyzed: the $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching mode, the $\nu\text{S}-\text{O}$ stretching mode, and the $\nu\text{C}-\text{O}$ stretching mode of the ester-linked sulfate group. Like the SGC spectra, the $\nu_{\text{s}}\text{S}=\text{O}$ symmetrical stretching mode ($1040\text{--}1080\text{ cm}^{-1}$) of SGG could not be used for analysis due to overlapping C-OH stretching bands from the sugar head group.

Any changes to the hydrogen bonding in the interfacial region, as a result of divalent cation interaction with the sulfate moiety in the polar head region of SGG, would be reflected in the SGG ester $\nu\text{C}=\text{O}$ stretching mode. The divalent cation interaction with the sulfate moiety in the polar head region of SGG would be expected to not only affect the hydrogen bonding in the interfacial region but also affect the dynamics of the hydrocarbon chains. Several absorption modes were analyzed to characterize the chain dynamics: the δCH_2 scissoring mode, the γCH_2 rocking mode, and the CH_3 bending mode. Also, the peak height intensity ratio (R) of δCH_2 and $\delta'\text{CH}_2$ was calculated as a function of pressure. The height intensity ratio (R') of $\gamma'\text{CH}_2$ and the "valley" component of the correlation field splitting of the CH_2 rocking mode was calculated as a function of pressure. These two parameters (R and R') were determined to aid in the spectroscopic identification of bilayer interdigitation (see below).

3.3.1 Cation Interaction with the Negatively-Charged Sulfate Moiety

The $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching mode absorbs in the region of 1200 to 1265 cm^{-1} (Kates, 1986). Figure 3.19A shows the infrared spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the frequency range of 1200 to 1240 cm^{-1} at ambient pressure. Analysis of this stretching mode for all four samples revealed a broad band comprised of two components: a high-frequency component (1222 cm^{-1}) and a low-frequency component (1208-1215 cm^{-1}). Fourier-deconvolution of the spectra was performed to separate the two overlapping components. The deconvolved spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the same region are shown in figure 3.19B. Upon addition of the divalent cations, the high-frequency component of the SGG $\nu_{\text{as}}\text{S}=\text{O}$ asymmetric stretching mode did not significantly change in frequency, intensity, or bandwidth. However, the intensity of the $\nu_{\text{as}}\text{S}=\text{O}$ low-frequency component was affected differently by each cation. Upon addition of both Ca^{2+} and Mg^{2+} , the intensity of this component increased. In contrast, the intensity of the $\nu_{\text{as}}\text{S}=\text{O}$ low-frequency component decreased upon the addition of Sr^{2+} .

Figure 3.19 - (A) The infrared spectra of hydrated SGG- Na^+ (—), SGG- Ca^{2+} (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (- - —) in the S=O asymmetric stretching region (1200-1240 cm^{-1}) at ambient pressure. (B) The Fourier-deconvolved spectra of hydrated SGG- Na^+ (—), SGG- Ca^{2+} (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (- - —) in the S=O asymmetric stretching region at ambient pressure.
Note: Spectra are baseline corrected.

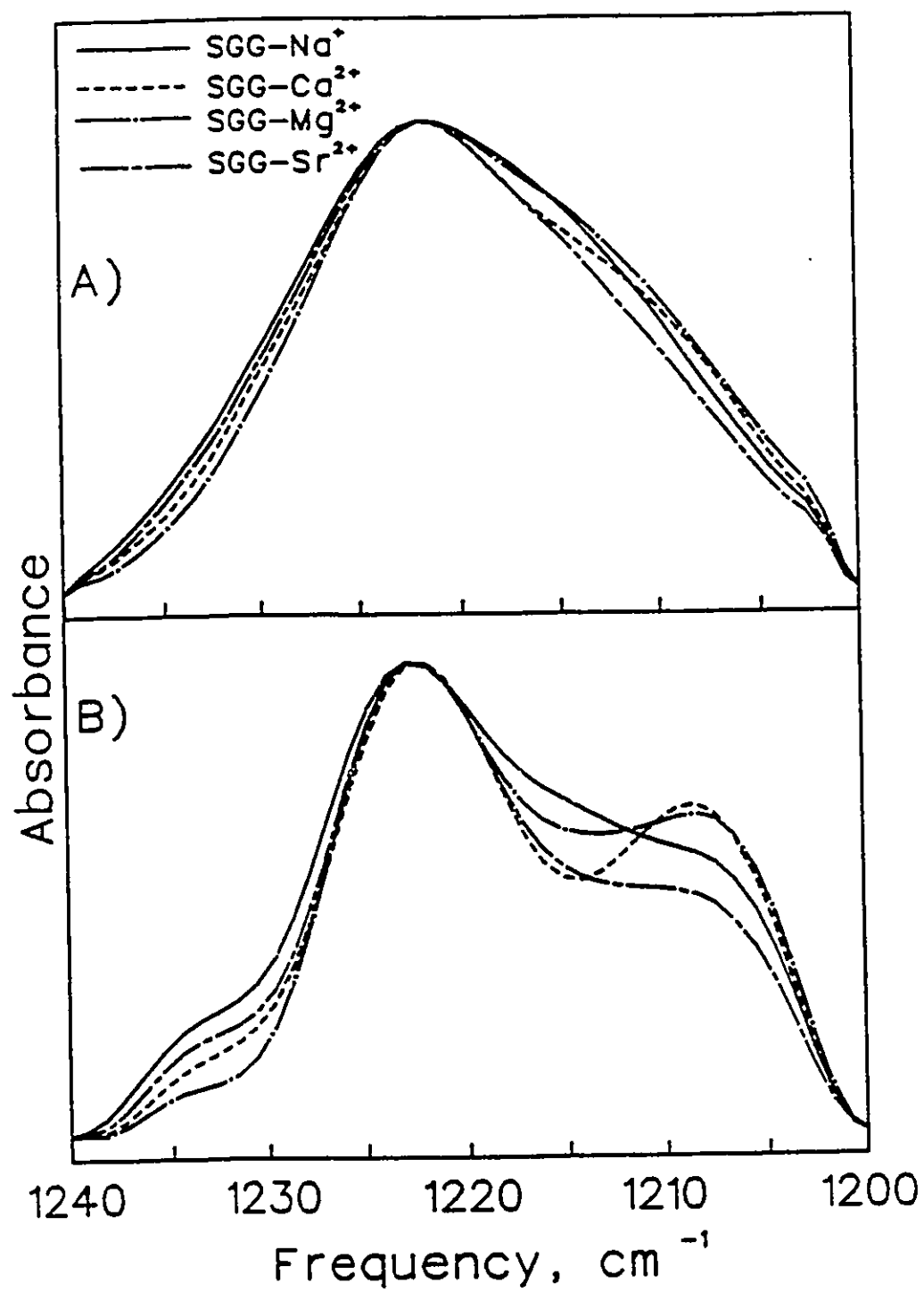


Figure 3.19

Figure 3.20 shows the spectra of solid SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the same region at ambient pressure. The band shape varied for all four cations, with the intensity of the $\nu_{\text{as}}\text{S}=\text{O}$ low-frequency component differing significantly for the three divalent cations. The addition of Ca^{2+} caused the greatest increase in intensity of the $\nu_{\text{as}}\text{S}=\text{O}$ low-frequency component, followed by Mg^{2+} . In contrast, Sr^{2+} addition significantly lowered the intensity of the $\nu_{\text{as}}\text{S}=\text{O}$ low-frequency component. The $\nu_{\text{as}}\text{S}=\text{O}$ high-frequency component was similar in frequency, intensity, and bandwidth for all the cations, with the exception of Ca^{2+} ; upon the addition of Ca^{2+} , the $\nu_{\text{as}}\text{S}=\text{O}$ high-frequency component decreased in frequency from 1222 to 1218 cm^{-1} , as determined from the derivative spectra (data not shown).

The $\nu\text{S}-\text{O}$ stretching mode absorbs in the region of 780 to 860 cm^{-1} (Kates, 1986). Figure 3.21 shows the spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} at ambient pressure in this frequency range. Upon addition of all three divalent cations, the peak absorption frequency of the $\nu\text{S}-\text{O}$ mode increased from 822 to 826 cm^{-1} . Moreover, the bandwidth of this mode became more narrow in the presence of each divalent cation.

Figure 3.22 presents the spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the $\nu\text{C}-\text{O}$ stretching region, which absorbs in the range of 950 to 1015 cm^{-1} (Kates, 1986). This $\nu\text{C}-\text{O}$ stretching mode absorbed at 989 cm^{-1} for all four samples. However, the bandwidth of the $\nu\text{C}-\text{O}$ mode increased slightly upon addition of all three divalent cations to SGG.

Figure 3.20 - The infrared spectra of solid SGG- Na^+ (—), SGG- Ca^{2+} (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (— - —) in the S=O asymmetric stretching region (1200-1240 cm^{-1}) at ambient pressure.

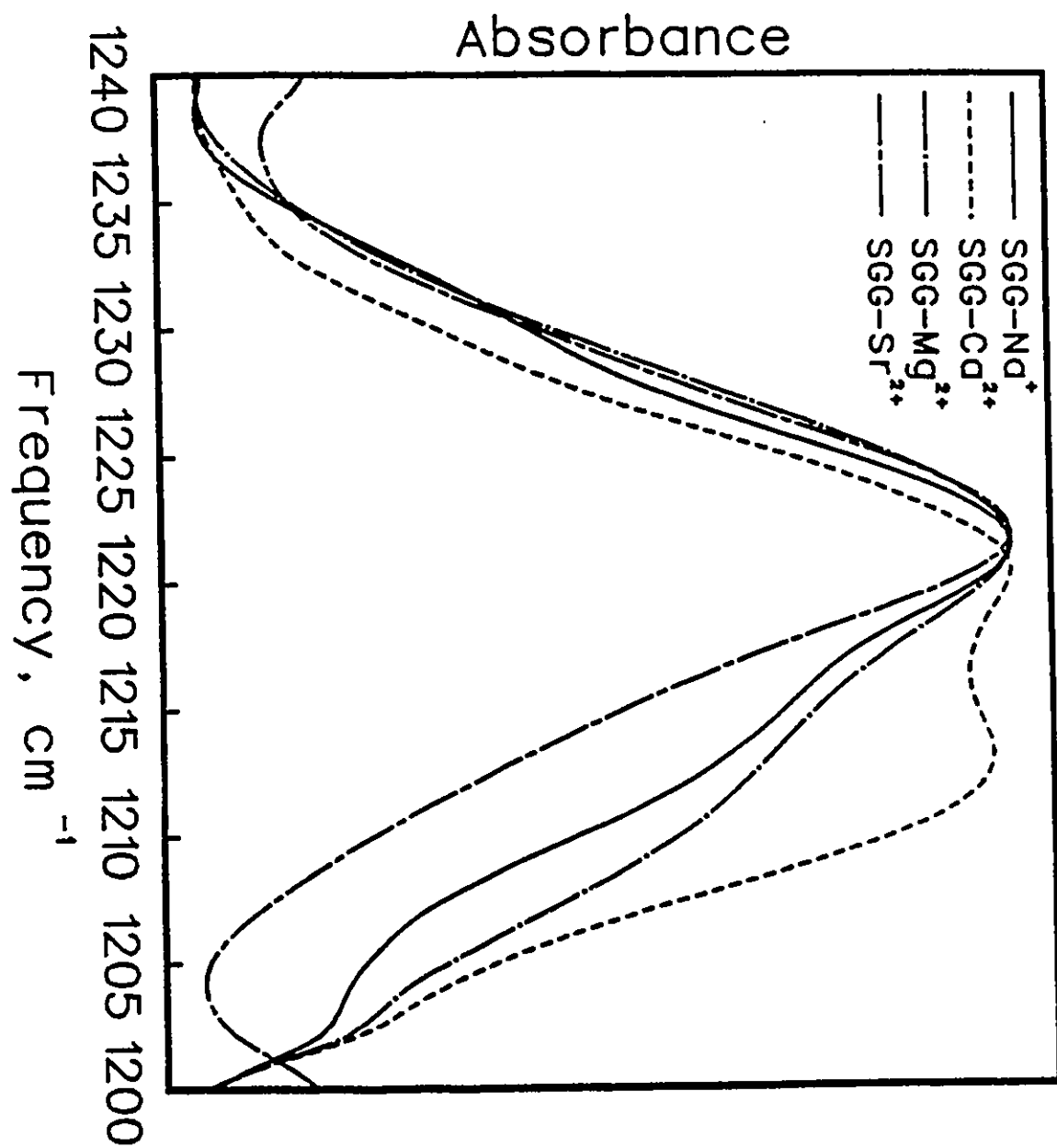


Figure 3.20

Figure 3.21 - The infrared spectra of hydrated SGG- Na^+ (—), SGG- Ca^{2+} (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (— - —) in the S-O stretching region ($780\text{-}860\text{ cm}^{-1}$) at ambient pressure.

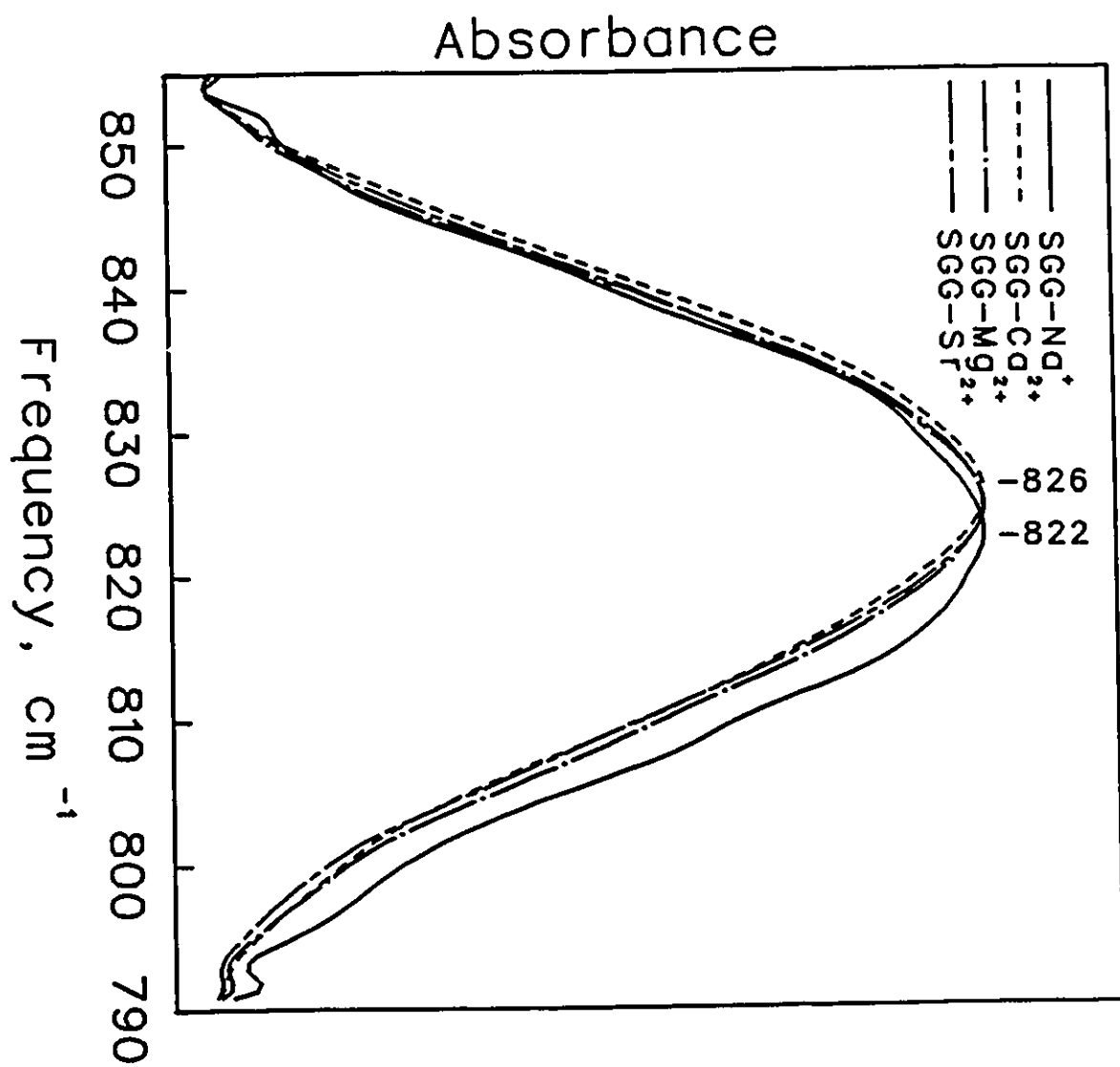


Figure 3.21

Figure 3.22 - The infrared spectra of hydrated SGG- Na^+ (—), SGG- Ca^{2+} (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (— - —) in the C-O stretching region ($965\text{-}1015\text{ cm}^{-1}$) at ambient pressure.

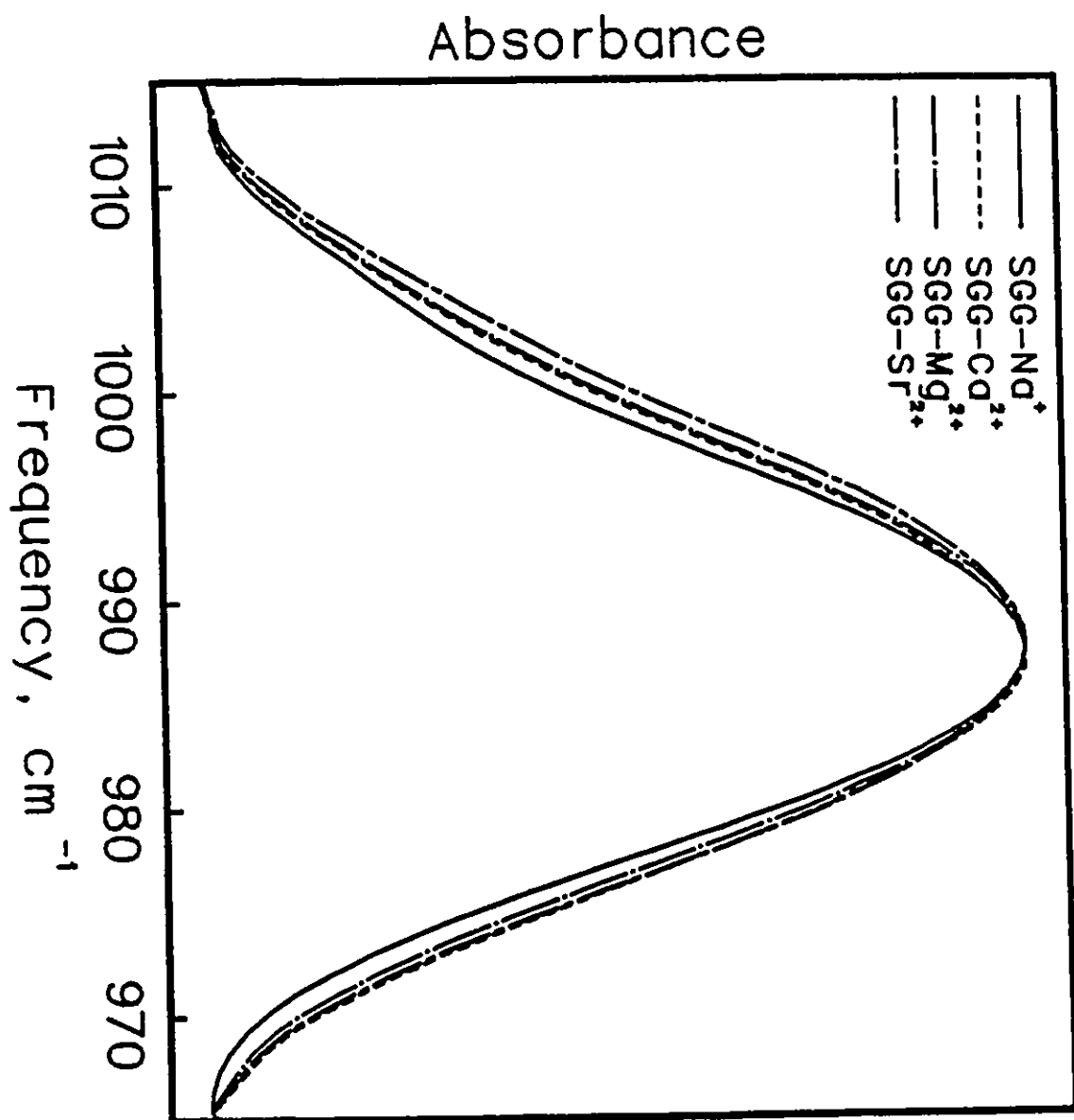


Figure 3.22

3.3.2 *The Interfacial Region (Ester Group)*

The $\nu\text{C}=\text{O}$ stretching mode absorbs in the frequency range of 1650 to 1750 cm^{-1} (Kates, 1986). Figure 3.23 shows the spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in this region at ambient pressure. For all four samples the $\nu\text{C}=\text{O}$ stretching mode absorbed as a broad band, which comprised of two components: a high-frequency component (1740 cm^{-1}) and a low-frequency component (1715 cm^{-1}). The $\nu\text{C}=\text{O}$ high-frequency component increased slightly in intensity and frequency (from 1735 to 1738 cm^{-1}) upon addition of each divalent cation. However, the intensity of the $\nu\text{C}=\text{O}$ low-frequency component was affected significantly and to different degrees upon divalent cation addition. The addition of Ca^{2+} decreased the intensity of the $\nu\text{C}=\text{O}$ low-frequency component to the greatest degree, followed by Sr^{2+} . In contrast, Mg^{2+} had little affect of the intensity of the low-frequency component of the ester $\nu\text{C}=\text{O}$ stretching mode. The broad band of SGG- Na^+ also has a small absorption at $\sim 1700 \text{ cm}^{-1}$ that appears absent from the other samples' spectra.

Figure 3.23 - The infrared spectra of hydrated SGG- Na^+ (—), SGG- Ca^{2+} , (----), SGG- Mg^{2+} (—·—), and SGG- Sr^{2+} (— - —) in the ester C=O stretching region (1650-1750 cm^{-1}) at ambient pressure.
Note: Spectra are baseline corrected.

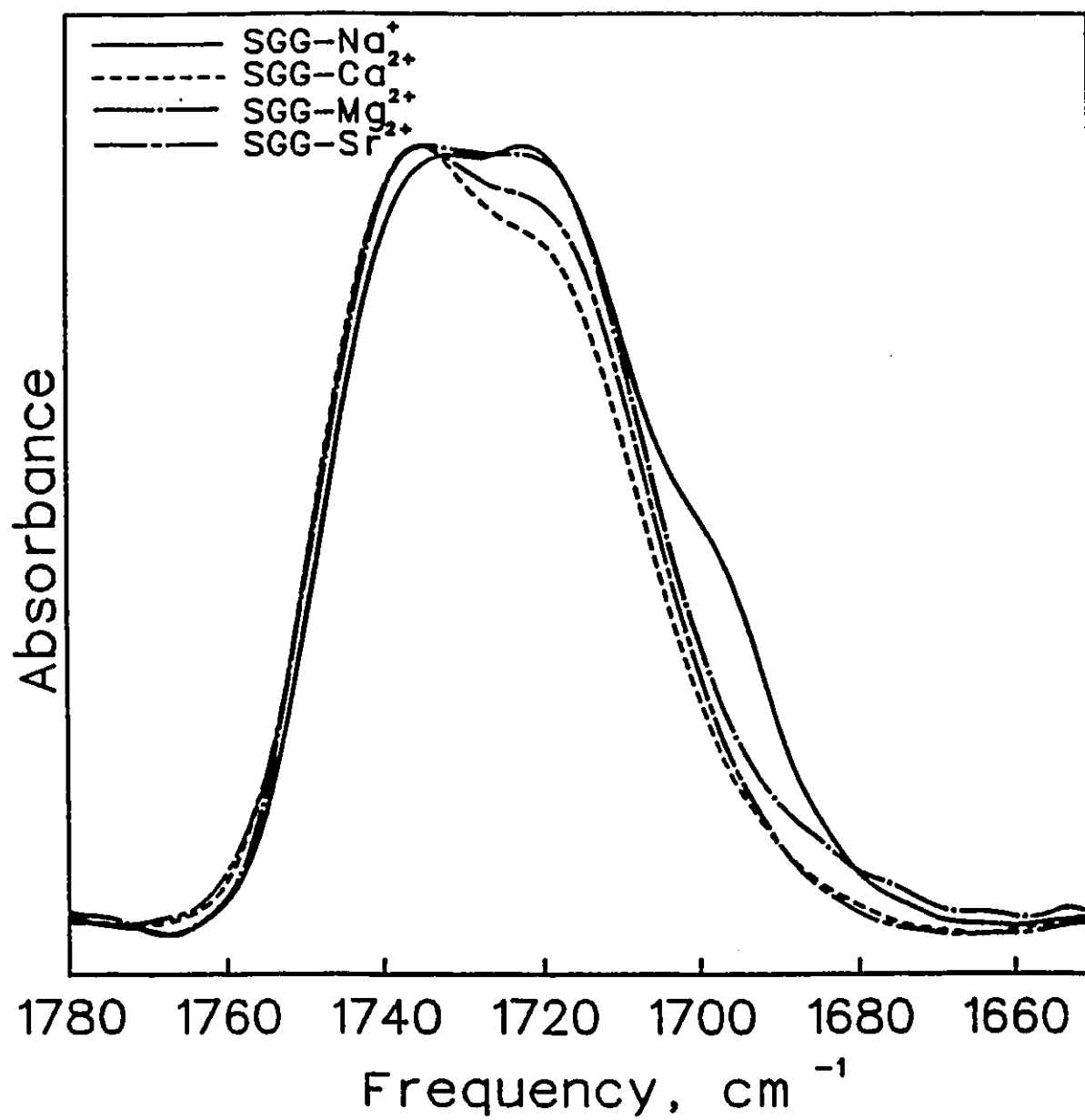


Figure 3.23

Figure 3.24 shows a plot of the pressure dependence of the frequency of the $\nu\text{C}=\text{O}$ low-frequency component for hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the frequency range of 1710 to 1720 cm^{-1} . The barotropic behaviour of this mode was similar in pattern for each divalent cation sample. For all samples, the peak absorption frequency of this mode initially decreased with increasing pressure; however above ~ 6 kbar, the peak absorption frequency remained constant with increasing pressure. The peak absorption frequencies for SGG- Ca^{2+} and SGG- Mg^{2+} , and SGG- Sr^{2+} (1714-1715 cm^{-1} at ambient pressure) were consistently lower than those of SGG- Na^+ (1717 cm^{-1} at ambient pressure), with a difference of $\sim 2\text{-}3$ cm^{-1} throughout the pressure range studied. Among the three divalent cations, the peak absorption frequencies for SGG- Mg^{2+} were slightly higher than those of SGG- Ca^{2+} and SGG- Sr^{2+} , with an approximate difference of 0.5-1 cm^{-1} for the entire pressure range.

Figure 3.24 - Pressure dependence of the frequencies of the low-frequency component of the ester C=O stretching mode ($1710\text{-}1720\text{ cm}^{-1}$) for hydrated SGG- Na^+ (+), hydrated SGG- Ca^{2+} (○), hydrated SGG- Mg^{2+} (●), and hydrated SGG- Sr^{2+} (X). *Frequencies were determined from third-order derivative spectra, calculated using a breakpoint of 0.3 in the Fourier domain.*

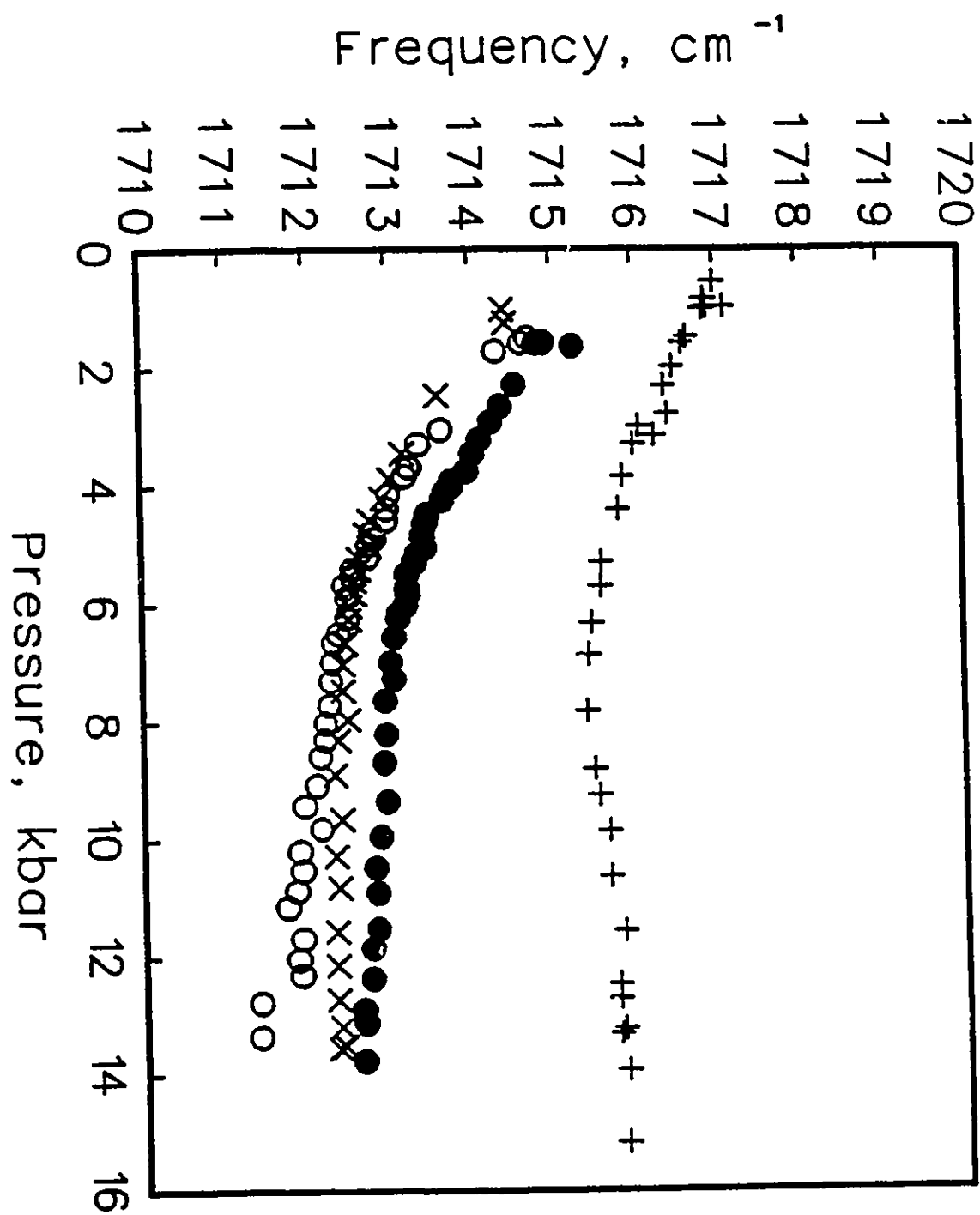


Figure 3.24

3.3.3 The Hydrocarbon Chain Structure

The δCH_2 scissoring mode absorbs in the region of 1430 to 1500 cm^{-1} (Kates, 1986). Figure 3.25 presents the stacked contour plots of the infrared spectra of hydrated SGG- Na^+ (A) and SGG- Ca^{2+} (B) in the δCH_2 scissoring region. Figure 3.26 presents the stacked contour plots of the infrared spectra of hydrated SGG- Mg^{2+} (A) and SGG- Sr^{2+} (B) in the δCH_2 scissoring region. At ambient pressure, the spectra of all four samples show an intense, narrow band at 1467 cm^{-1} . However, the SGG- Na^+ spectra differed in its barotropic behaviour when compared to SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} . Visual inspection reveals that the correlation field splitting in SGG- Na^+ increased more rapidly with increasing pressure, producing a pronounced, wide "valley" between the two component bands. In contrast, the $\delta'\text{CH}_2$ correlation field splitting band in the spectra of SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} , initially appeared as only a broad shoulder on the high-frequency side of the δCH_2 band, which gradually gained intensity until a well-defined correlation field component band $\delta'\text{CH}_2$ became evident. The peak height intensities of $\delta'\text{CH}_2$ and δCH_2 and their ratios are tabulated as a function of pressure in tables 3.9 to 3.12 for SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} , respectively. Figure 3.27 compares the pressure dependence of the peak height intensity $I_{\delta'\text{CH}_2}/I_{\delta\text{CH}_2}$ (R) ratio for all four samples. The SGG- Na^+ R ratio clearly shows a different pressure profile in comparison to SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} . The SGG- Na^+ R ratio only increased slightly from 0.96 to 1.01 over the entire pressure range. In contrast, the R ratios for SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} increased significantly from 0.97 to 1.15 with increasing pressure.

Figure 3.25 - Stacked contour plots of the infrared spectra of the CH₂ scissoring mode (1430-1500 cm⁻¹) of (A) hydrated SGG-Na⁺ and (B) hydrated SGG-Ca²⁺. The numbers on the right of the plots are pressure values in kbar.

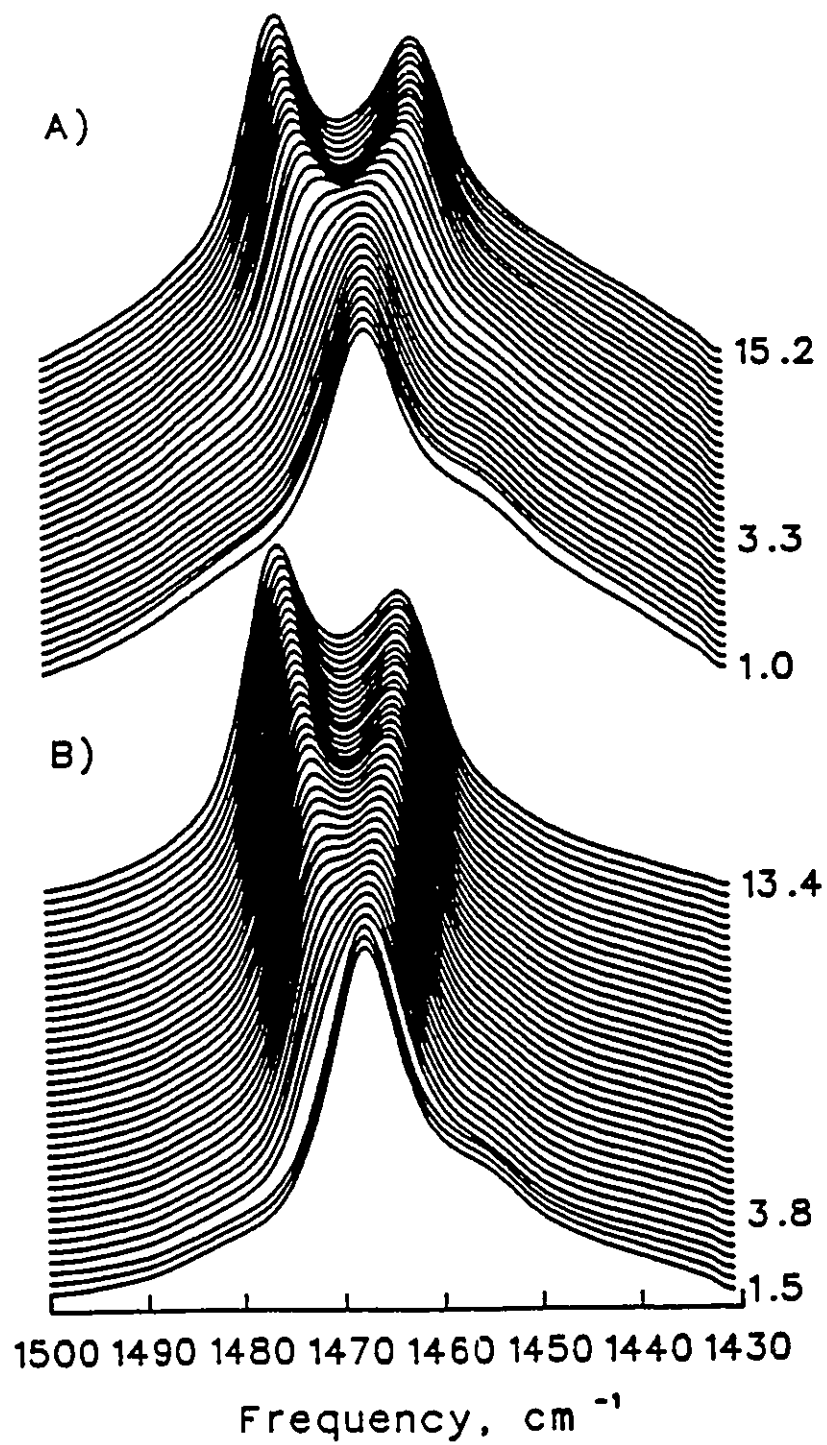


Figure 3.25

Table 3.9 Height Intensities of $\delta'\text{CH}_2$ and δCH_2 bands and $I\delta'\text{CH}_2/I\delta\text{CH}_2$ ratio (R) over increasing pressure for SGG- Na^+

Pressure (kbar)	Height Intensity of $\delta'\text{CH}_2$ Band ($I\delta'\text{CH}_2$) [†]	Height Intensity of δCH_2 Band ($I\delta\text{CH}_2$) [†]	Ratio (R) of $I\delta'\text{CH}_2/I\delta\text{CH}_2$
6.32	0.72	0.76	0.96
6.86	0.72	0.75	0.96
7.83	0.72	0.74	0.97
8.81	0.69	0.72	0.97
9.25	0.69	0.71	0.96
9.87	0.68	0.71	0.96
10.63	0.67	0.69	0.96
11.57	0.66	0.68	0.98
12.47	0.66	0.66	0.99
12.71	0.65	0.65	1.00
13.22	0.65	0.65	1.00
13.31	0.64	0.64	1.00
13.95	0.64	0.63	1.01
15.10	0.61	0.60	1.01

[†] $\delta'\text{CH}_2$ and δCH_2 are the high- and low-frequency component of the correlation field splitting of the CH_2 scissoring mode, respectively.

NRC software (RAMOP) was used to determine the peak intensities.

Table 3.10 Height intensities of $\delta'\text{CH}_2$ and δCH_2 bands and $I\delta'\text{CH}_2/I\delta\text{CH}_2$ ratio (R) over increasing pressure for SGG- Ca^{2+}

Pressure (kbar)	Height Intensity of $\delta'\text{CH}_2$ Band ($I\delta'\text{CH}_2$)	Height Intensity of δCH_2 Band ($I\delta\text{CH}_2$)	Ratio (R) of $I\delta'\text{CH}_2/I\delta\text{CH}_2$
6.23	0.66	0.68	0.97
6.50	0.66	0.67	0.98
6.66	0.67	0.68	0.99
6.99	0.66	0.66	1.00
7.30	0.69	0.68	1.02
7.72	0.67	0.66	1.02
8.03	0.67	0.66	1.02
8.32	0.67	0.65	1.02
8.59	0.67	0.66	1.02
9.08	0.67	0.65	1.02
9.42	0.67	0.66	1.02
9.82	0.69	0.68	1.02
10.20	0.68	0.65	1.04
10.53	0.69	0.65	1.06
10.87	0.69	0.65	1.07
11.15	0.70	0.65	1.08
11.68	0.70	0.64	1.09
12.04	0.71	0.64	1.10
12.30	0.72	0.65	1.10
12.77	0.71	0.64	1.11
13.35	0.71	0.64	1.11

Figure 3.26 - Stacked contour plots of the infrared spectra of the CH₂ scissoring mode (1430-1500 cm⁻¹) of (A) hydrated SGG-Mg²⁺ and (B) hydrated SGG-Sr²⁺. The numbers on the right of the plots are pressure values in kbar.

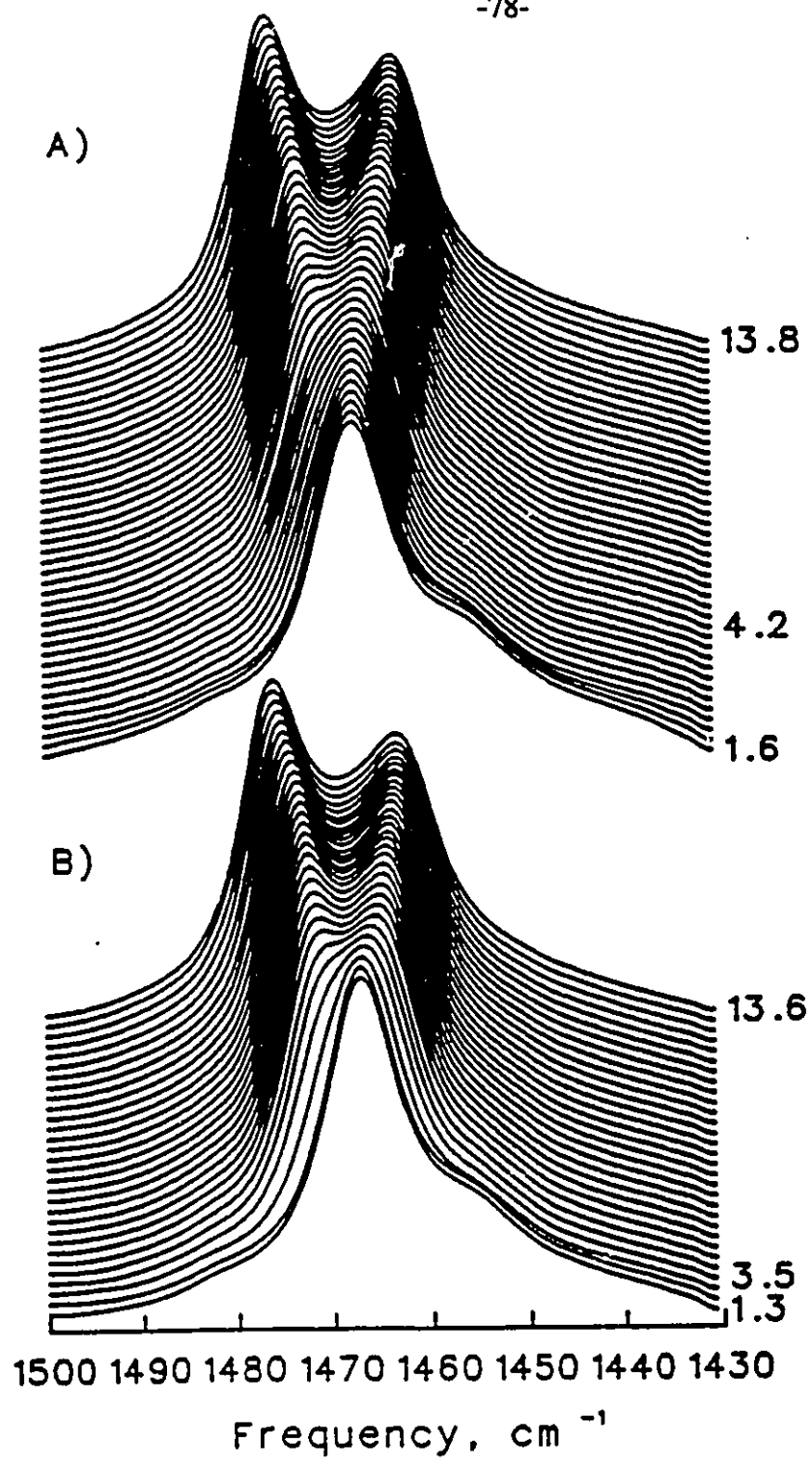


Figure 3.26

Table 3.11 Height intensities of $\delta'\text{CH}_2$ and δCH_2 bands and $I\delta'\text{CH}_2/I\delta\text{CH}_2$ ratio (R) over increasing pressure for SGG-Mg²⁺

Pressure (kbar)	Height Intensity of $\delta'\text{CH}_2$ Band ($I\delta'\text{CH}_2$)	Height Intensity of δCH_2 Band ($I\delta\text{CH}_2$)	Ratio (R) of $I\delta'\text{CH}_2/I\delta\text{CH}_2$
6.02	0.58	0.61	0.95
6.15	0.58	0.60	0.97
6.24	0.59	0.60	0.97
6.55	0.59	0.60	0.98
6.99	0.59	0.59	1.00
7.26	0.59	0.59	1.00
7.64	0.60	0.60	1.00
8.21	0.59	0.59	1.00
8.68	0.59	0.59	1.00
9.34	0.60	0.60	1.00
9.95	0.60	0.59	1.01
10.47	0.61	0.59	1.03
10.90	0.62	0.59	1.04
11.51	0.63	0.59	1.06
11.84	0.64	0.59	1.07
12.37	0.63	0.58	1.07
12.90	0.63	0.58	1.08
13.12	0.64	0.58	1.08
13.76	0.62	0.57	1.07

Table 3.12 Height intensities of $\delta'\text{CH}_2$ and δCH_2 bands and $I\delta'\text{CH}_2/I\delta\text{CH}_2$ ratio (R) over increasing pressure for SGG- Sr^{2+}

Pressure (kbar)	Height Intensity of $\delta'\text{CH}_2$ Band ($I\delta'\text{CH}_2$)	Height Intensity of δCH_2 Band ($I\delta\text{CH}_2$)	Ratio (R) of $I\delta'\text{CH}_2/I\delta\text{CH}_2$
5.40	0.81	0.84	0.97
5.60	0.81	0.83	0.97
5.83	0.81	0.83	0.97
6.04	0.81	0.83	0.98
6.25	0.82	0.83	0.99
6.65	0.83	0.83	1.00
7.01	0.84	0.83	1.01
7.46	0.84	0.82	1.02
7.97	0.85	0.83	1.03
8.31	0.85	0.83	1.03
8.91	0.85	0.82	1.03
9.66	0.85	0.82	1.04
10.29	0.84	0.82	1.03
10.81	0.86	0.81	1.05
11.57	0.88	0.81	1.09
12.14	0.89	0.80	1.11
12.72	0.90	0.80	1.13
13.19	0.90	0.79	1.14
13.50	0.90	0.79	1.14
13.55	0.90	0.79	1.14

Figure 3.27 - Pressure dependence of the peak height intensity ratio R ($I\delta^1CH_2/I\delta CH_2$) for hydrated SGG- Na^+ (+), hydrated SGG- Ca^{2+} (O), hydrated SGG- Mg^{2+} (●), and hydrated SGG- Sr^{2+} (X).

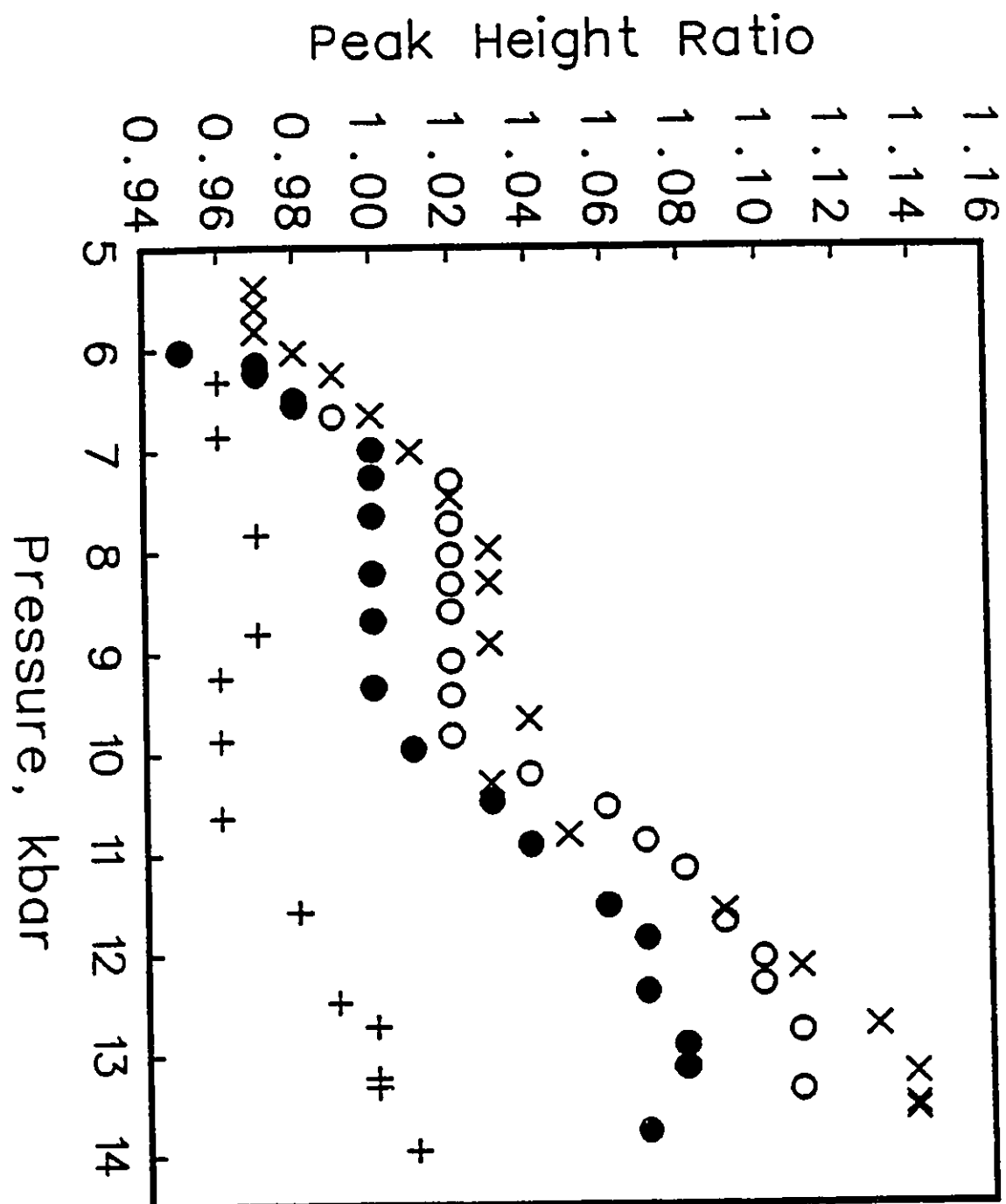


Figure 3.27

Figure 3.28 shows a plot of the pressure dependence of the δCH_2 mode frequencies of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} . The correlation field splitting pressure of SGG increased in the presence of all three divalent cations. SGG- Mg^{2+} had the highest splitting pressure of 4.2 kbar, followed by SGG- Ca^{2+} (3.8 kbar). The presence of Sr^{2+} only caused a small increase in the splitting pressure from 3.3 kbar (SGG- Na^+) to 3.5 kbar. Also, the correlation field splitting of the δCH_2 mode was slightly more rapid and greater in magnitude for SGG- Na^+ than for SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} . This wider splitting concurs with the wide "valley" noted in the stacked contour plots of SGG- Na^+ (Figure 3.25A).

Figure 3.28 - Pressure dependence of the frequencies of the CH₂ scissoring mode (1460-1478 cm⁻¹) for hydrated SGG-Na⁺ (+), hydrated SGG-Ca²⁺ (○), hydrated SGG-Mg²⁺ (●), and hydrated SGG-Sr²⁺ (X). The numbers in parentheses are the splitting pressures in kbar. *Frequencies were determined from third-order derivative spectra, calculated using a breakpoint of 0.95 in the Fourier domain.*

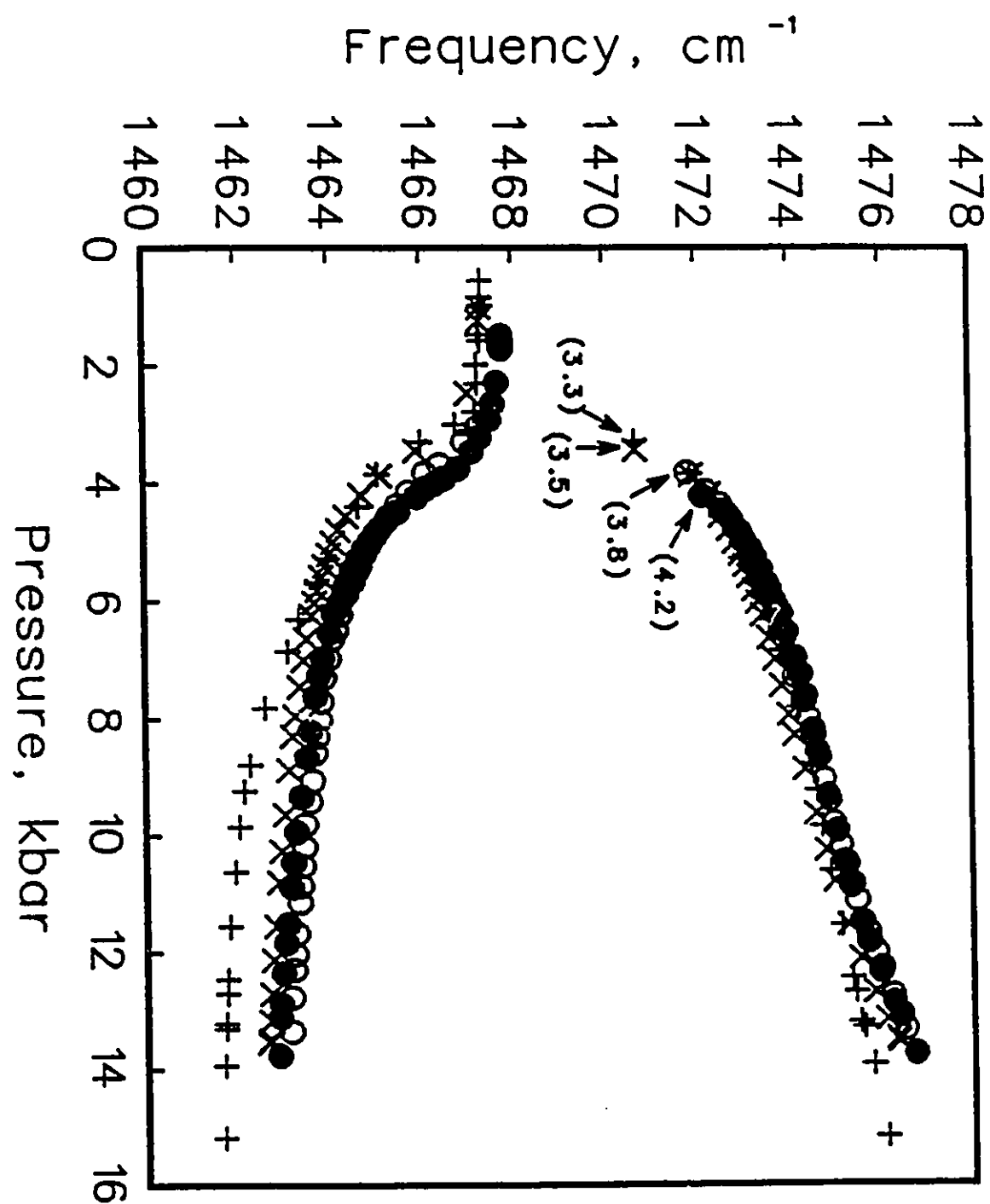


Figure 3.28

The γCH_2 rocking mode absorbs in the frequency range of 700 to 750 cm^{-1} (Kates, 1986). Figures 3.29, 3.30, 3.31 and 3.32 respectively show the stacked contour plots of the infrared spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the frequency range of 675 to 840 cm^{-1} . To obtain accurate peak heights, a stable baseline was required over increasing pressure. The frequency range in which the CH_2 rocking mode absorbs is quite crowded, with many shifting bands (over increasing pressure). The spectra had to be plotted with starting and ending points that corresponded to absorption minima over the entire pressure range. It was therefore necessary to include the big bands above and below 740 cm^{-1} to satisfy the requirement of a stable baseline. With the absorption minima at the starting and ending points, NRC software was used to determine the extent of absorption of the relevant peaks.

Similar to the SGG δCH_2 scissoring mode, with increasing pressure, a correlation field splitting (arrow) of the SGG γCH_2 rocking mode occurred. At ambient pressure, the spectra of all four samples show an intense, narrow peak absorption at 720 cm^{-1} . However, the barotropic behaviour of the SGG- Na^+ spectra differed from that of SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} spectra. The intensity of the correlation field splitting band $\gamma'\text{CH}_2$ in SGG- Na^+ increased more rapidly with increasing pressure, producing a deep "valley" between the two component bands. In contrast, the "valley" produced by the correlation field splitting of the SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} spectra was comparatively more shallow than that of SGG- Na^+ . The height intensities of $\gamma'\text{CH}_2$ and the "valley" component and their $I_{\gamma'\text{CH}_2}/I_{\text{"valley"}}$ (R') ratios are tabulated as a function of pressure in tables 3.13 to 3.16 for SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} ,

respectively. Ratios were determined by dividing the height intensity of the γ' CH₂ band by the height intensity of the "valley" component. Figure 3.33 compares the pressure dependence of the height intensity R' ratio for all four samples. The SGG-Na' R' ratio increased significantly from 1.0 to 2.4 in the pressure range studied. In contrast, the height intensity R' ratios of SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ only gradually increased with pressure, increasing from 1.0 to 1.6.

Figure 3.29 - Stacked contour plots of the infrared spectra of the CH₂ rocking mode (700-750 cm⁻¹) in the frequency range 675-840 cm⁻¹ for hydrated SGG-Na⁺. Arrow marks correlation field splitting band, δ' CH₂.

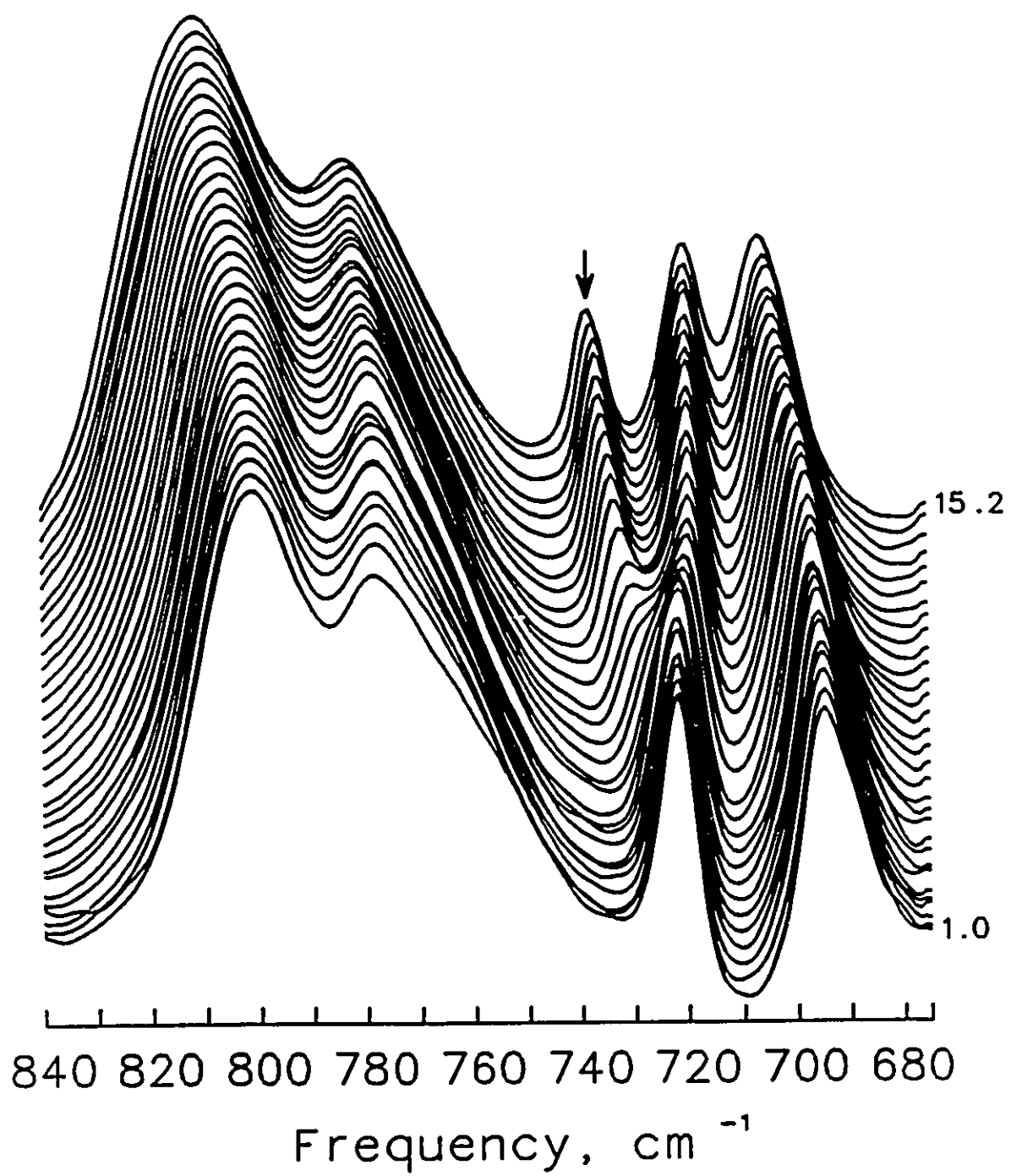


Figure 3.29

Table 3.13 Height intensities of γ' CH₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Na⁺

Pressure (kbar)	Height Intensity of γ' CH ₂ Band ($I_{\gamma'CH_2}$) [†]	Height Intensity of "Valley" Component ($I_{\text{"valley"}}$)	Ratio (R') of $I_{\gamma'CH_2}/I_{\text{"valley"}}$
6.86	0.070	0.065	1.08
7.83	0.079	0.059	1.35
8.81	0.085	0.050	1.70
9.25	0.086	0.052	1.67
9.87	0.091	0.049	1.87
10.63	0.095	0.048	1.98
11.57	0.098	0.047	2.06
12.47	0.097	0.044	2.21
12.71	0.101	0.044	2.29
13.22	0.105	0.044	2.39
13.31	0.105	0.045	2.33
13.95	0.104	0.044	2.38
15.18	0.103	0.044	2.33

[†] γ' CH₂ is the high-frequency component of the correlation field splitting of the CH₂ rocking mode.

NRC software (RAMOP) was used to determine the peak intensities.

Figure 3.30 - Stacked contour plots of the infrared spectra of the CH₂ rocking mode (700-750 cm⁻¹) in the frequency range 675-840 cm⁻¹ for hydrated SGG-Ca²⁺. Arrow marks correlation field splitting band, δ' CH₂.

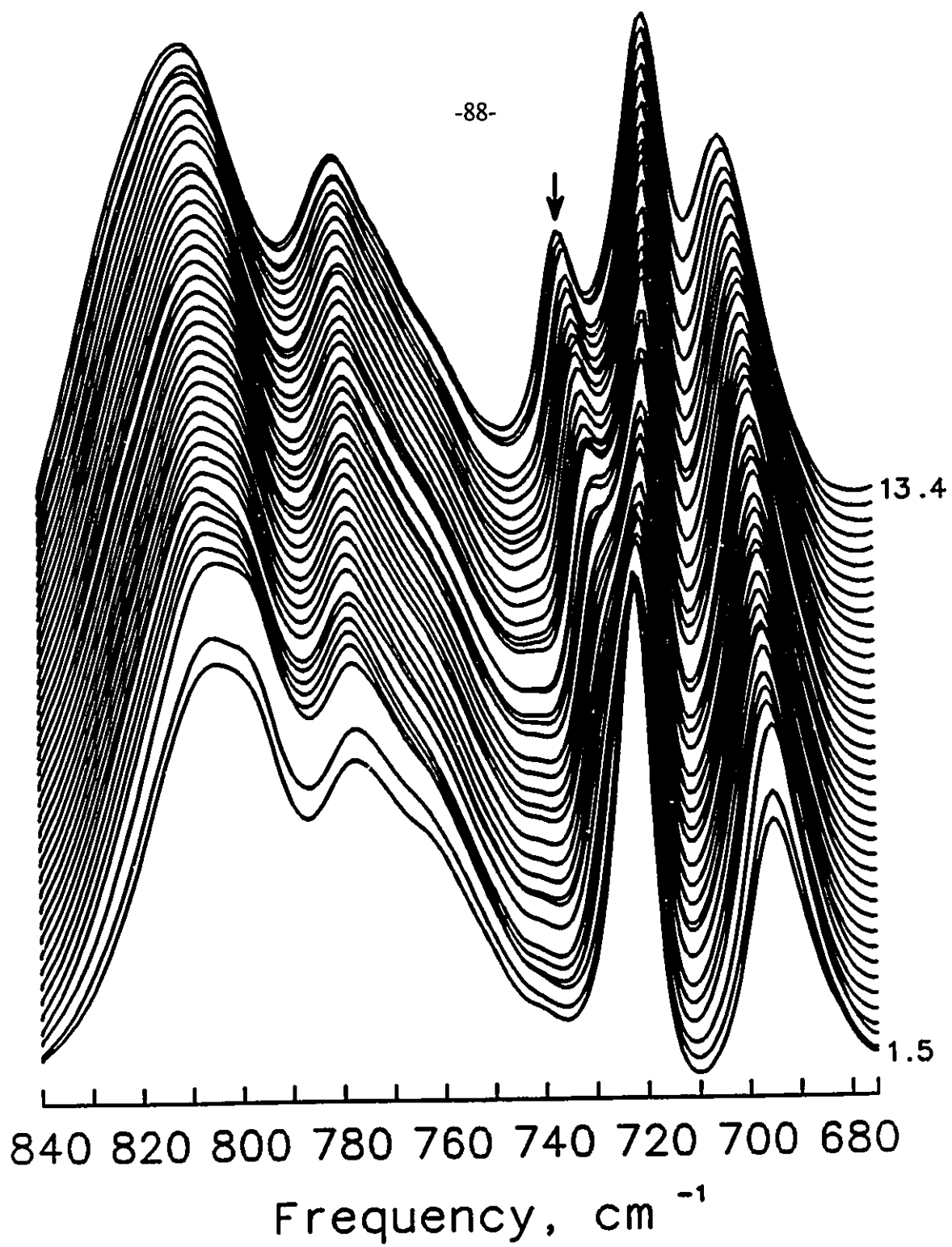


Figure 3.30

Table 3.14 Height intensities of γ' CH₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Ca²⁺

Pressure (kbar)	Height Intensity of γ' CH ₂ Band ($I_{\gamma'CH_2}$)	Height Intensity of "Valley" Component ($I_{\text{"valley"}}$)	Ratio (R') of $I_{\gamma'CH_2}/I_{\text{"valley"}}$
8.59	0.130	0.123	1.06
9.08	0.130	0.121	1.07
9.42	0.135	0.123	1.10
9.82	0.144	0.128	1.13
10.20	0.127	0.111	1.14
10.53	0.128	0.111	1.15
10.87	0.125	0.109	1.15
11.15	0.126	0.108	1.17
11.68	0.127	0.105	1.21
12.04	0.126	0.102	1.23
12.30	0.138	0.112	1.23
12.77	0.128	0.102	1.26
13.35	0.128	0.097	1.32

Figure 3.31 - Stacked contour plots of the infrared spectra of the CH₂ rocking mode (700-750 cm⁻¹) in the frequency range 675-840 cm⁻¹ for hydrated SGG-Mg²⁺. Arrow marks correlation field splitting band, δ' CH₂.

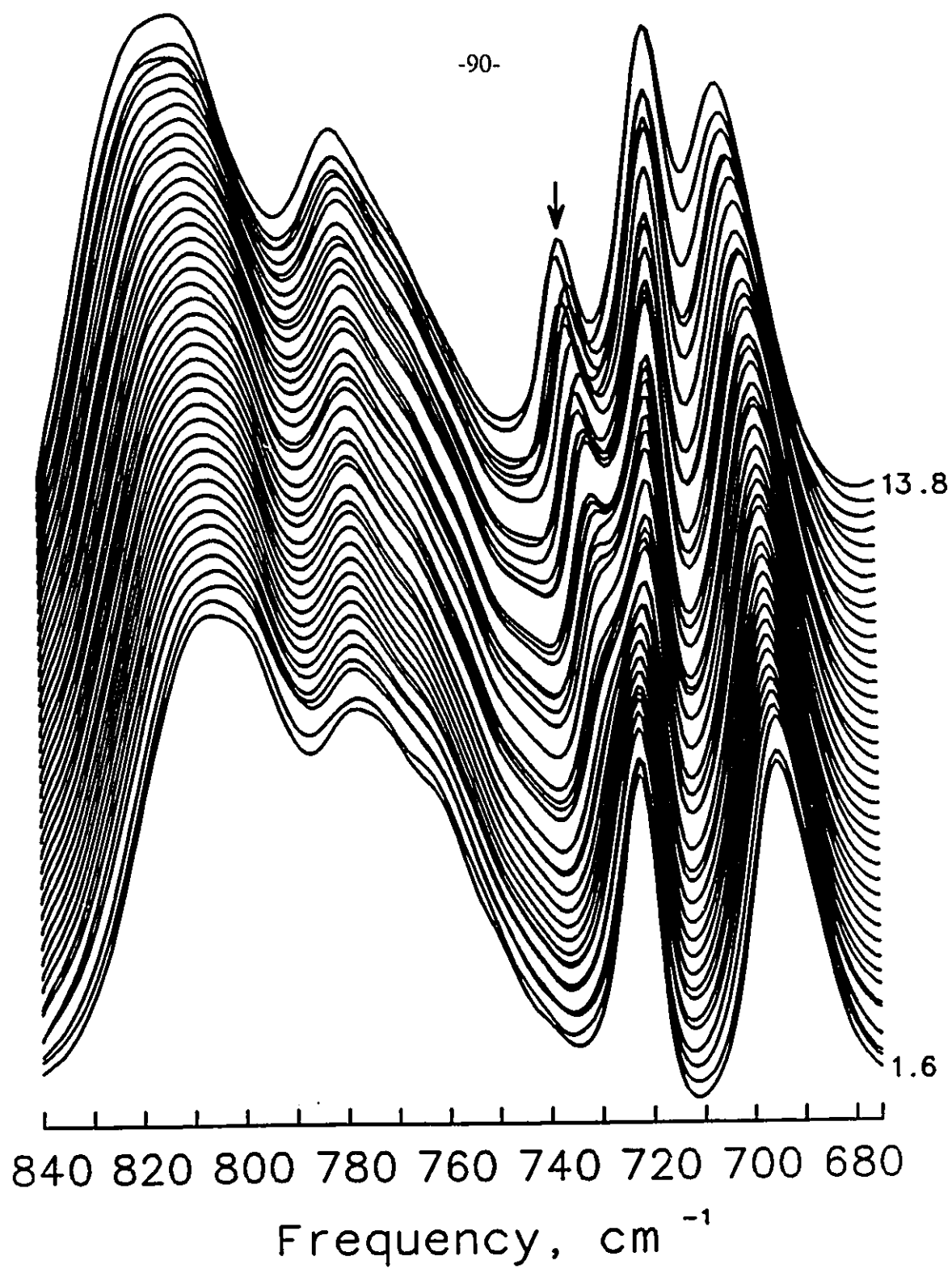


Figure 3.31

Table 3.15 Height intensities of γ' CH₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Mg²⁺

Pressure (kbar)	Height Intensity of γ' CH ₂ Band ($I_{\gamma'CH_2}$)	Height Intensity of "Valley" Component ($I_{\text{"valley"}}$)	Ratio (R') of $I_{\gamma'CH_2}/I_{\text{"valley"}}$
7.26	0.100	0.094	1.06
7.64	0.120	0.111	1.08
8.21	0.110	0.099	1.11
8.68	0.107	0.095	1.13
9.34	0.124	0.107	1.16
9.95	0.102	0.080	1.26
10.47	0.101	0.078	1.29
10.90	0.116	0.092	1.27
11.51	0.124	0.094	1.33
11.84	0.126	0.093	1.36
12.37	0.103	0.069	1.48
12.90	0.102	0.068	1.51
13.12	0.128	0.088	1.45
13.76	0.108	0.067	1.62

Figure 3.32 - Stacked contour plots of the infrared spectra of the CH₂ rocking mode (700-750 cm⁻¹) in the frequency range 675-840 cm⁻¹ for hydrated SGG-Sr²⁺. Arrow marks correlation field splitting band, δ'CH₂.

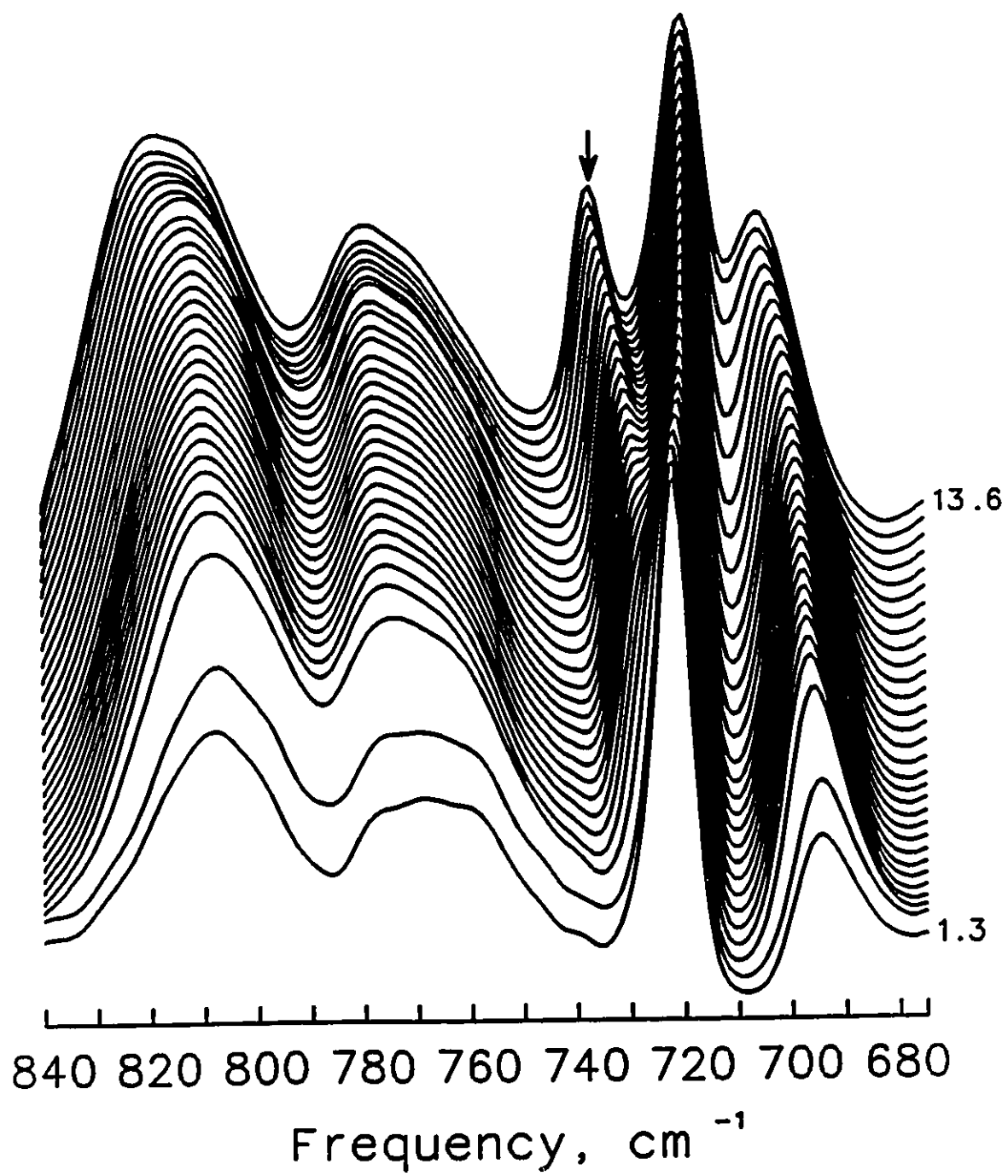


Figure 3.32

Table 3.16 Height intensities of γ' CH₂ band and "valley" component and $I_{\gamma'CH_2}/I_{\text{"valley"}}$ ratio (R') over increasing pressure for SGG-Sr²⁺

Pressure (kbar)	Height Intensity of γ' CH ₂ Band ($I_{\gamma'CH_2}$)	Height Intensity of "Valley" Component ($I_{\text{"valley"}}$)	Ratio (R') of $I_{\gamma'CH_2}/I_{\text{"valley"}}$
7.01	0.163	0.152	1.07
7.46	0.164	0.148	1.11
7.97	0.163	0.145	1.13
8.31	0.163	0.142	1.14
8.91	0.164	0.139	1.18
9.66	0.167	0.135	1.24
10.29	0.166	0.132	1.26
10.81	0.164	0.128	1.28
11.57	0.168	0.123	1.37
12.14	0.168	0.119	1.42
12.72	0.164	0.115	1.43
13.19	0.168	0.113	1.49
13.50	0.168	0.111	1.52
13.55	0.169	0.110	1.53

Figure 3.33 - Pressure dependence of the height intensity ratio R' ($I_{\gamma'CH_2}/I''_{\text{valley}}$) for hydrated SGG- Na^+ (+), hydrated SGG- Ca^{2+} (O), hydrated SGG- Mg^{2+} (●), and hydrated SGG- Sr^{2+} (X).

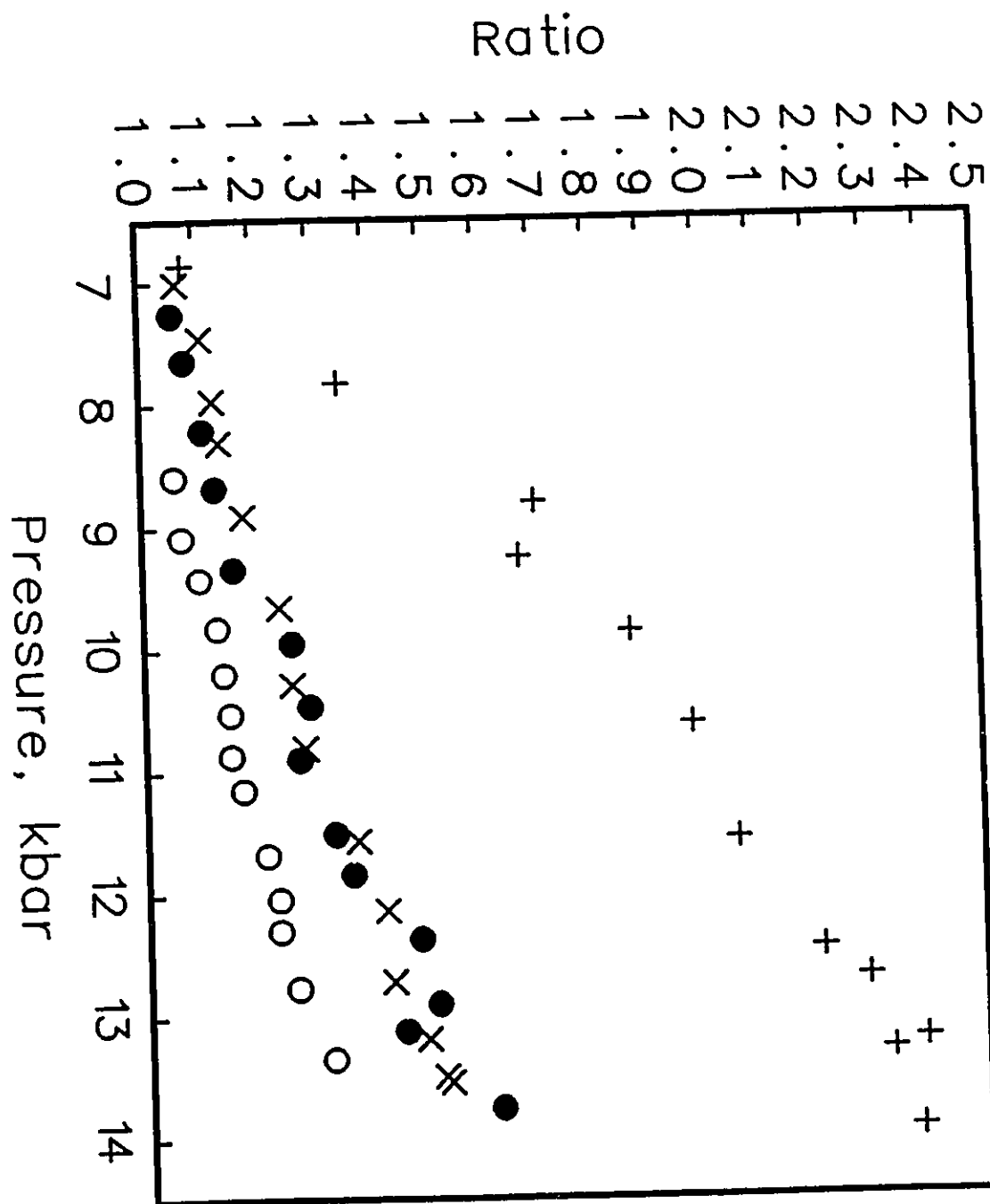


Figure 3.33

The CH_3 symmetrical bending mode of the hydrocarbon chains absorbs in the region of 1355 to 1395 cm^{-1} (Bellamy, 1975). Figure 3.34 shows the plot of the pressure dependence of this mode for SGG-Na^+ , SGG-Ca^{2+} , SGG-Mg^{2+} , and SGG-Sr^{2+} in the frequency range of 1379 to 1383 cm^{-1} . For all samples, the peak absorption frequency increased with increasing pressure. However, the peak absorption frequencies of SGG-Na^+ (1379.5 cm^{-1} at ambient pressure) are consistently lower than those of SGG-Ca^{2+} , SGG-Mg^{2+} , and SGG-Sr^{2+} , with a difference of 0.5 to 3 cm^{-1} throughout the pressure range studied. In addition, the peak absorption frequencies of SGG-Sr^{2+} are intermediate between those of SGG-Na^+ and $\text{SGG-Ca}^{2+}/\text{SGG-Mg}^{2+}$, throughout the pressure range studied.

Figure 3.34 - Pressure dependence of the frequencies of the CH₃ bending mode (1379-1383 cm⁻¹) for hydrated SGG-Na⁺ (+), hydrated SGG-Ca²⁺ (○), hydrated SGG-Mg²⁺ (●), and hydrated SGG-Sr²⁺ (X). *Frequencies were determined from third-order derivative spectra, calculated using a breakpoint of 0.3 in the Fourier domain.*

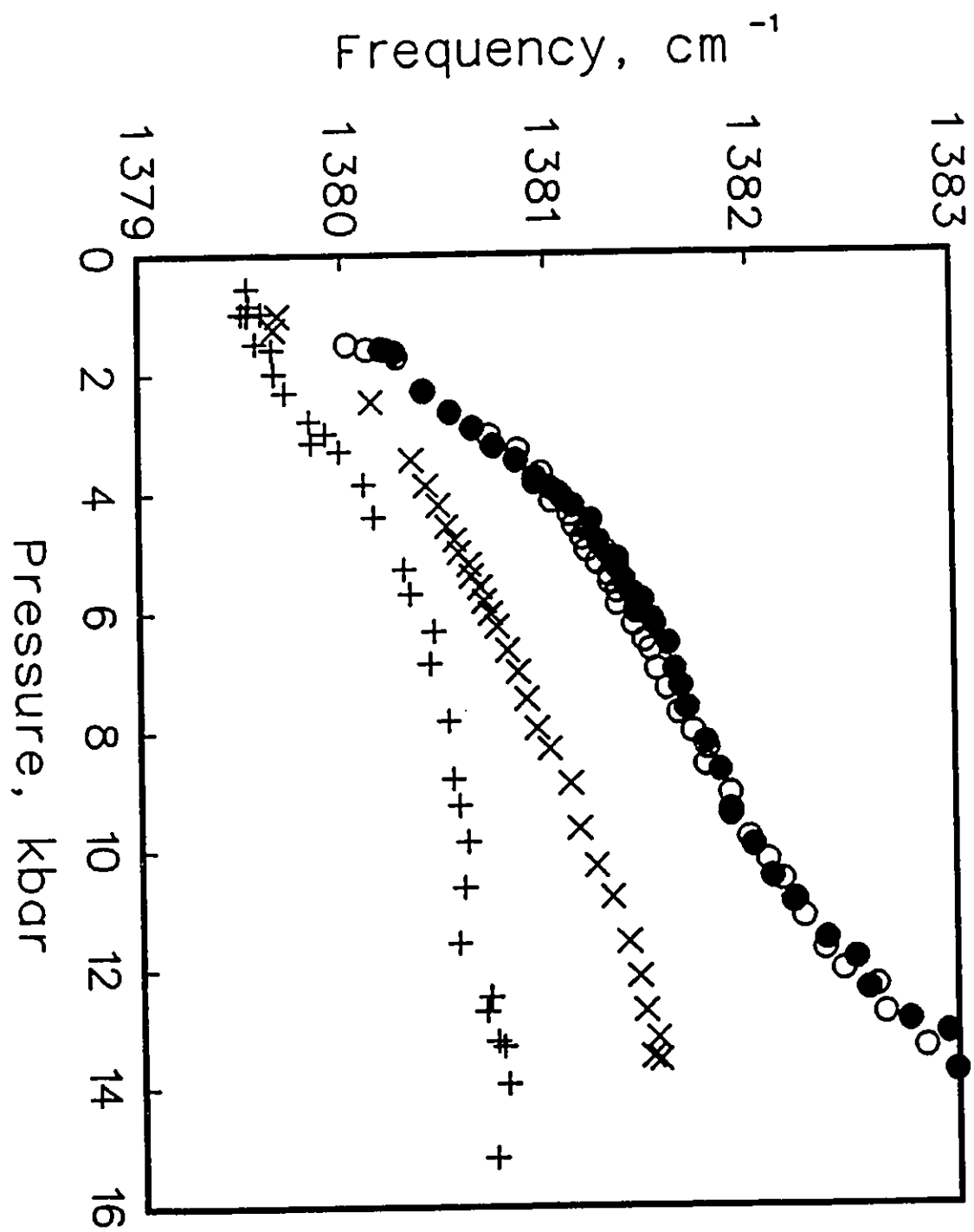


Figure 3.34

CHAPTER FOUR

DISCUSSION

4.1 Characterization of SGG, SGC, and GC

The identity and purity of SGG were verified using TLC and infrared spectroscopy. Kornblatt et al. (1974) demonstrated by TLC that SGG and GG were the major glycolipids of rat testes. These two glycolipids could be separated from other testicular lipids by silicic acid column chromatography; SGG and GG were eluted from the column by acetone (Kornblatt et al., 1974). Similar procedures were used in this study to prepare SGG and GG from the ram testes. The results indicated that elution of the testicular lipids chromatographed onto the silicic acid column with CHCl_3 :acetone (1:1) prior to acetone elution effectively separated the faster-migrating GG from the slower-migrating SGG. As a result, only acetone elution followed by cold acetone precipitation (to fractionate out any contaminating neutral lipids (Kates, 1986)) was necessary for SGG purification. The purity of SGG was confirmed by ascending TLC using the solvent system of CHCl_3 :MeOH:H₂O:conc. ammonia (65:25:3:1 v/v) (Kornblatt, 1979) (Figure 3.1). Positive staining by α -naphthol verified the presence of a sugar moiety in all glycolipid samples. The R_f values obtained for SGG, SGC, nGC, α GC, and mGC were very similar, if not identical, to those obtained by Gadella, Colenbrander, van Golde, and Lopes-Cardozo (1992) using the same solvent system. The single TLC band obtained for SGG, albeit broad, provided further evidence of its high purity. The broad band can be accounted for by the various SGG alkyl subclasses, as determined by methanolysis/acetolysis of SGG and subsequent GLC analysis (Tables

3.7 and 3.8, Figure 3.8, see below). Finally, the identity and purity of SGG was further confirmed by infrared spectroscopic analysis. The infrared spectrum of SGG extracted from ram testes (Figure 3.2B) was very similar to that of boar testis SGG (Figure 3.2A) described by Ishizuka et al. (1973).

Characterization of the acyl subclass of SGG revealed that the major acyl group was palmitate (16:0, ~95%), with small amounts of stearate (18:0, ~3%), myristate (14:0, <1%), and myristoleate (14:1, ~1%) (Table 3.8). The findings of this study are very similar to those of Kornblatt et al. (1974), who characterized the acyl and alkyl moieties of rat testis SGG. They found that the acyl group is 86-98% palmitate (16:0) with small amounts of stearate (18:0) and oleate (18:1) present. They characterized the alkyl moiety as being 71-83% chimyl alcohol (16:0), with the remainder being batyl alcohol (18:0). We found the ram testis SGG alkyl group to be more heterogeneous in its molecular subclasses, with the major alkyl group being ~65% chimyl alcohol (16:0). Minor alkyl subclasses include tetradecanol (14:0, ~7%), pentadecanol (15:1, ~7%), pentadecanol (15:0, ~2%), and heptadecanol (17:0, ~1%) (Table 3.8).

The characteristics of ram testes SGG alkyl subclasses were very similar to those described by Ishizuka et al. (1975) for boar testis SGG. The alkyl group of boar testes SGG is predominantly chimyl alcohol (16:0, ~77%) with minor amounts of tetradecanol (14:0, ~15%) and pentadecanol (15:0, ~3%). It is striking that the acyl and alkyl groups of SGG from several species are predominantly C16:0 (Kornblatt et al., 1974; Ishizuka et al., 1973; Suzuki, Ishizuka, Veta, and Yamakawa, 1973; Knapp, Kornblatt, Schachter, and Murray, 1973).

4.2 Infrared Analysis of SGC and GC

4.2.1 Calcium Binding to the Negatively-Charged Sulfate Moiety

Comparison of mGC and SGC spectra verified the position of the S=O asymmetric stretching mode at 1220 cm^{-1} (Figure 3.9) (Kates, 1986). Upon addition of Ca^{2+} , the S=O asymmetric stretching mode of SGC decreased in frequency from 1219 to 1215 cm^{-1} , reflecting a decrease in the S=O bond energy. Ionic binding of Ca^{2+} to the negatively charged sulfate group would result in a transfer of electrons from the sulfate group to the Ca^{2+} ion and a corresponding decrease in the S=O bond strength. Therefore, our results clearly indicate the Ca^{2+} bound tightly to the sulfate group of SGC. This conclusion agrees with the finding of Quinn and Sherman (1971), using the surface radioactivity technique, that SGC binds tightly to Ca^{2+} .

To determine whether Ca^{2+} binding to the SGC sulfate group affected the overall conformation of the sulfate moiety, the C-O stretching mode of the ester-linked sulfate group was analyzed. The frequency of this stretching mode was verified by comparison of the SGC spectra to mGC, which lacked any major absorption around 990 cm^{-1} . The small decrease in the C-O stretching frequency from 992 to 991 cm^{-1} indicated that the C-O bond energy was slightly weakened upon Ca^{2+} binding (Figure 3.10). However, the bandwidth of this mode was seemingly not affected by Ca^{2+} binding, suggesting that the conformational order of the sulfate group is not significantly affected. The stability of a conformation is determined by its bond angles, angles of rotation, and even bond lengths (Morrison and Boyd, 1983). Due to the divalent nature of Ca^{2+} , it is likely that

the cation was serving as a bridge between two SGC neighbouring head groups. In fact, Boggs and colleagues have suggested that Ca^{2+} most likely bridges adjacent SGC molecules but without inducing closer packing because of the large head group (Boggs, Koshy, and Rangaraj, 1984). Such crosslinking would put conformational strains on the sulfate group and likely weaken the C-O bond that links the sulfate moiety of the sugar head group.

Finally, the O-H and N-H stretching modes of SGC were analyzed to exclude the possibility of electrostatic interactions between Ca^{2+} and the sugar O-H groups or amide N-H group (Figure 3.11). An electrostatic interaction of Ca^{2+} with the O-H or N-H moieties would result in a slight transfer in electron density and a decrease in the O-H/N-H bond strength, which would be much lower in magnitude than that caused by ionic binding or hydrogen bonding. Spectroscopically, we obtained the opposite results; upon addition of Ca^{2+} to SGC, the broad overlapping O-H and N-H stretching modes increased slightly in absorption frequency, reflecting an increase in the O-H and N-H bond strength, and a decrease in the strength of hydrogen bonding between adjacent SGC molecules. The decrease in hydrogen bonding strength was likely due to an increase in the intermolecular distance between neighbouring SGC molecules upon Ca^{2+} binding. The maximum absorption by each sample is well below 1.0 in this frequency range indicating that the data is relevant with no interfering water vapour absorption.

It was not necessary to study H_2O sample to verify Ca^{2+} binding to the SGC sulfate moiety. The solid samples were prepared by lyophilizing the SGC- Ca^{2+} lipid multilayers followed by purging with N_2 . By preparing the solid samples in such a

manner, Ca^{2+} bound to the SGC sulfate moieties while in dispersion. Solid samples were analyzed to remove the factor of hydration, thus demonstrating Ca^{2+} binding more clearly in the SGC IR spectra. Since solid samples were only analyzed to demonstrate cation interaction with the sulfate moiety of SGC and not to characterize any other lipid interaction, there were no major pitfalls related to the solid study. However, the author acknowledges that the solid structure of a lipid multilayer and its interaction can differ significantly from its hydrated structure. This, however, would not change the fact that the sulfate group binds to cation.

4.2.2 The Interfacial Region (Amide Group)

Since the SGC used in this study was a mixture of α -hydroxy ($\sim 60\%$) and non-hydroxy ($\sim 40\%$) fatty acids, it was important to determine first the impact of fatty acid α -hydroxylation on hydrogen bonding before studying the effects of Ca^{2+} binding. To achieve this, the spectra of α GC, nGC, and mGC were compared.

The hydrogen bonding of crystalline α GC has been well characterized by Pascher and colleagues using X-ray crystallography (Pascher, 1976; Pascher and Sundell, 1977; Nyholm, Pascher, and Sundell, 1990). Their findings were used, in part, in the interpretation of the spectroscopic results of the α GC, nGC, and mGC study. Pascher and colleagues have found that the amide group of crystalline α GC is highly involved in hydrogen bonding and has a significant effect on the conformational structure of the molecule and the interactions with neighbouring molecules. The amide $\text{C}=\text{O}$ group is

hydrogen bonded with both the O-H group of the sphingosine chain and the 3-OH of the sugar head group from neighbouring molecules. The hydrogen atom of the amide nitrogen forms a bifurcated intramolecular hydrogen bond with the oxygen of the fatty acid α -hydroxyl group and the oxygen of the glycosidic linkage. These interactions restrict the orientation possibilities of any ligands and give rise to a shovel-shaped conformation of the α GC molecule (Pascher, 1976; Pascher and Sundell, 1977; Nyholm, Pascher, and Sundell, 1990).

In contrast, an absence of the fatty acid α -hydroxyl group would likely allow the interfacial region to be more mobile and likely result in an alteration of its conformation and interactions with neighbouring molecules. We compared the spectra of nGC, α GC, and mGC to determine what influence the α -hydroxyl group had on the hydrogen bonding of the amide group.

The infrared spectra of the amide I C=O stretching mode of α GC, nGC, and mGC are shown in figure 3.12. The relatively high peak absorption frequency of the nGC amide I band, at 1646 cm^{-1} , is indicative of a weakly hydrogen bonded C=O group. In contrast, the α GC amide I band absorbed at 1627 cm^{-1} , a frequency associated with a strongly hydrogen-bonded C=O group. The results suggest that the presence of the α -hydroxyl group gave rise to a stronger hydrogen bonding of the amide C=O. As determined by Pascher and colleagues, the locked conformation of the α GC molecule allows the amide C=O to strongly hydrogen bond with both the hydroxyl group of the sphingosine chain and the 3-OH of the sugar head group from neighbouring molecules (Pascher and Sundell, 1977). In contrast, in the absence of the α -hydroxyl group, the

amide group would no longer be in a locked conformation and would thus be more mobile. This increased mobility would allow the nGC amide C=O group to be positioned in a energetically more favourable conformation and not necessarily in the proximity of hydrogen bonding donors, thus resulting in weaker hydrogen bonding. The mGC spectrum, which shows two components in its absorption mode, is simply a composite of the α GC and nGC spectra. One interesting note to make about the mGC spectrum, however, is the noticeably lower intensity of its low-frequency component (α GC) compared to its high-frequency component (nGC), in spite of the fact that α GC and nGC constitute 60% and 40% of the mGC mixture, respectively. As mentioned in Appendix One, the absorption of infrared energy by a bond depends upon its dipole moment. The mere presence of the α -hydroxyl group beside the C=O bond in α GC would result in a shift in electron density, a consequential decrease in the C=O dipole moment, and in turn, spectroscopically, a decrease in the intensity of the amide C=O stretching mode.

The original spectrum (A) and third-order derivative spectra (B) of the amide I C=O stretching mode of SGC- Na^+ and SGC- Ca^{2+} are shown in figure 3.13. Both the original and derivative spectra were baseline corrected (i.e. the low-frequency component intensities were made the same) for comparison purposes. Let it be noted that comparison is made between the relative intensities of each component and not their absolute intensities. The spectra of SGC, which is a mixture of α -hydroxy (~60%) and non-hydroxyl (~40%) fatty acid was comprised of two components, as expected. Comparison of the SGC derivative spectra (Figure 3.13B) with the α GC and nGC

spectra (Figure 3.12) suggests that the high-frequency component, at 1647 cm^{-1} , originated mostly from the non-hydroxy SGC, whereas the low-frequency component, at 1619 cm^{-1} , was from α -hydroxy SGC.

The presence of a sulfate group affected the amide C=O stretching mode of both SGC molecular species. Sulfation of the 3-OH group of the sugar caused the amide C=O group of the non-hydroxy component of SGC- Na^+ to hydrogen bond somewhat more weakly than in nGC, as reflected by the small increase in vibrational frequency from 1646 to 1647 cm^{-1} (Figures 3.12 and 3.13). This slight decrease in the strength of hydrogen bonding is most likely the result of an increased intermolecular distance due to charge repulsion contributed by the negatively charged sulfate group. The decrease in frequency of the C=O stretching mode from 1627 to 1619 cm^{-1} of the α -hydroxy component (Figures 3.12 and 3.13) indicates that the presence of the sulfate group caused the amide C=O group of SGC- Na^+ to hydrogen bond even more strongly than α GC. The increase in hydrogen bonding strength is likely due to conformational changes that have allowed hydrogen bonding donors to come closer in proximity to the amide C=O group. In contrast to the non-hydroxy SGC component, the negatively charged sulfate group appeared not to cause an increase in intermolecular distance for the α -hydroxy component. Most likely, the stronger intermolecular hydrogen bonding of the amide group in the α -hydroxy SGC component may prevent any increase in intermolecular distance and as a result reduce the effect of the sulfate group.

The binding of Ca^{2+} increased the C=O stretching mode frequency of the non-hydroxy SGC from 1647 to 1651 cm^{-1} (Figure 3.13B). This apparent decrease in

hydrogen bonding strength is likely due to an increase in the intermolecular distance between neighbouring SGC molecules. Moreover, the intensity of the non-hydroxy SGC band decreased upon Ca^{2+} binding, reflecting a decreased dipole moment in the $\text{C}=\text{O}$ bond. This decrease in polarity would weaken any hydrogen bonding that the amide $\text{C}=\text{O}$ group was involved in as suggested by the increase in vibrational frequency. The vibrational frequency of the α -hydroxy SGC component was not affected by Ca^{2+} binding. Once again, it appears that the stronger hydrogen bonding of the α -hydroxy SGC amide group prevented any increase in the intermolecular distance, and consequently the structure of the amide group was not affected by Ca^{2+} binding. In contrast, Ca^{2+} binding weakened the hydrogen bonding network of the interfacial region of the SGC molecular species that possesses the non-hydroxy fatty acid.

4.2.3 The Hydrocarbon Chain Structure

Before analyzing the effect of Ca^{2+} binding on the SGC hydrocarbon chain dynamics, the influence of fatty acid hydroxylation was first investigated. The CH_2 scissoring mode of nGC, α GC, and mGC was analyzed over increasing pressure (Figures 3.14A,B and 3.15). The barotropic behaviour of this mode is sensitive to the orientational and conformational order of the lipid's hydrocarbon chains. To reiterate what was stated in Appendix One, increasing pressure dampens the chain fluctuations and motions and it eventually induces a correlation field splitting of the vibrational mode. If the chains are more orientationally disordered, a higher pressure is required to induce the correlation field splitting. This correlation field splitting occurs when the

hydrocarbon chains become nearly perpendicular (i.e., nonequivalent) to each other. The magnitude of the correlation field splitting pressure can be used to determine the order/disorder dynamics of the hydrocarbon chains in lipid multilayers (Wong, 1985, 1988, In Press).

At ambient pressure, the CH_2 scissoring mode of nGC, α GC, and mGC absorbed as an intense, narrow band at 1467 cm^{-1} (Figure 3.14A,B and 3.15), indicating that for all three samples, the hydrocarbon chains were fully extended under ambient conditions. However, due to significant reorientational fluctuations and twisting and torsion motions, the chains were still disordered. Comparison of the stacked contour plots of the CH_2 scissoring mode of nGC and α GC reveals two significantly different barotropic behaviours (Figure 3.14A,B). In the α GC spectra (Figure 3.14B), a correlation field splitting was induced at pressure values above 5.4 kbar. In contrast, the correlation field splitting band of the CH_2 scissoring mode is barely noticeable in the nGC spectra (Figure 3.14A), suggesting that the neighbouring hydrocarbon chains of the lipid molecules had an approximately equivalent parallel orientation. However, dampening of the reorientational fluctuations of the hydrocarbon chains still occurred in nGC with increasing pressure, as indicated by the narrowing of the band with increasing pressure (Figure 3.14A). Finally, the barotropic behaviour of the CH_2 scissoring mode of mGC (Figure 3.15) appears to be similar to that of α GC (Figure 3.14B) with the exception of a different magnitude of correlation field splitting pressure (2.4 kbar). However, the value of the α GC splitting pressure (~ 5.4 kbar) cannot be accurately determined from the data due to a significant jump (due to experimental error) in the pressure just prior

to the correlation field splitting. Consequently and unfortunately, the splitting pressure of α GC and mGC cannot be accurately compared. Differential scanning calorimetry studies by Boggs (1987) have shown, however, that the α -hydroxyl group in SGC and GC increases the stability of the gel phase, presumably because of additional hydrogen bonds. Given these findings, we would expect that the correlation field splitting pressure of α GC would be lower than that of mGC.

With the exception of the correlation field splitting pressure values, the stacked contour plots of the CH_2 scissoring mode of SGC-Na^+ and SGC-Ca^{2+} exhibited very similar barotropic behaviour (Figure 3.16). The narrow, intense absorption at 1467 cm^{-1} indicated that the hydrocarbon chains of SGC-Na^+ and SGC-Ca^{2+} were fully extended at ambient pressure.

The plot of the pressure dependence of the CH_2 scissoring mode frequencies of mGC, SGC-Na^+ , and SGC-Ca^{2+} (Figure 3.17) clearly illustrates the differences in their correlation field splitting pressures. The high splitting pressure of SGC-Na^+ (4.3 kbar) in comparison to mGC (2.4 kbar) indicated that the hydrocarbon chains of SGC were significantly more disordered than those of mGC. The increased disorder may arise from the decreased intermolecular hydrogen bonding of the amide group of the non-hydroxy SGC (Figures 3.12 and 3.13). Moreover, the presence of the negatively charged sulfate group would increase the intermolecular distance of the non-hydroxy fatty acid component (Figures 3.12 and 3.13). Consequently, the space between the hydrocarbon chains of neighbouring lipid molecules would be larger, and higher pressure would be required to provide the same compression on the hydrocarbon chains than that needed

for mGC. In contrast, the hydrocarbon chains of the α -hydroxy fatty acid component were probably not affected by the negatively charged sulfate group, given its negligible effect on the amide group of the interfacial region (Figure 3.13). Therefore, the difference in chain disorder between SGC- Na^+ and mGC was most likely due to the effect of the negatively charged sulfate group on the non-hydroxy SGC- Na^+ component. This conclusion agrees with previous observations from differential scanning calorimetry studies by Boggs and colleagues which revealed that SGC with an α -hydroxy fatty acid has a higher order-disorder transition temperature, thus indicating that it has a more ordered gel state than SGC with a non-hydroxy fatty acid (Koshy and Boggs, 1983; Boggs, Koshy, and Rangaraj, 1984).

The higher splitting pressure of SGC- Ca^{2+} (6.3 kbar), in comparison to SGC- Na^+ (4.3 kbar), clearly indicated that the hydrocarbon chains of SGC became more disordered upon Ca^{2+} binding. This agrees with the observation that a divalent cation such as Ca^{2+} causes a decrease in the order-disorder transition temperature of various sulfatides, indicative of an increase in hydrocarbon chain disorder (Boggs et al., 1984). Therefore, the binding of Ca^{2+} to the sulfate group of SGC resulted in a further weakening of the intermolecular hydrogen bonding and an increase in the intermolecular distance. Again, this difference in chain disorder is most likely due to the non-hydroxy component since the Ca^{2+} binding to SGC weakened the hydrogen bonding of the amide group of only the non-hydroxy species and not the α -hydroxy species (Figure 3.13). However, no conclusions can be made on the exact influence the α -hydroxyl group has on the Ca^{2+} binding effects until a Ca^{2+} binding study is done using the two purified SGC molecular

species with and without an α -hydroxyl group separately.

To determine whether the SGC bilayer midplane was affected by Ca^{2+} binding, the CH_3 bending mode was analyzed (Figure 3.18). The pressure dependence of this mode was analyzed to emphasize the difference in absorption frequency between SGC- Na^+ and SGC- Ca^{2+} . The interactions between the methyl groups on the hydrocarbon chains of opposing monolayers (at the bilayer midplane) are extremely strong (Wong and Mantsch, 1988). If these interactions are weakened, the absorption frequency of the CH_3 bending mode would decrease. The barotropic behaviour of SGC- Na^+ and SGC- Ca^{2+} demonstrates that the methyl group interactions were enhanced with increasing pressure and reduced volume. The consistently lower absorption frequency of SGC- Ca^{2+} indicates that the methyl group interactions were weakened upon Ca^{2+} binding to SGC. Presumably, this is from an increase in hydrocarbon chain disorder.

4.3 Infrared Analysis of SGG

4.3.1 Cation Interaction with the Negatively-Charged Sulfate Moiety

In contrast to the Ca^{2+} binding study of solid SGC, we found that the divalent cations did not bind tightly to the sulfate group of hydrated SGG. Rather, they electrostatically interacted with the sulfate moiety. As opposed to ionic binding, electrostatic interactions do not result in a significant transfer of electron density, instead, the dipole moment of the bond is affected. Spectroscopically, a change in a bond's dipole moment results in a change in its absorption mode intensity (Colthup et al., 1975).

This is what we see in the case of the S=O asymmetric stretching mode of SGG (Figure 3.19). Upon Ca^{2+} and Mg^{2+} addition, the relative intensity of this mode increased, reflecting a strong electrostatic interaction between $\text{Ca}^{2+}/\text{Mg}^{2+}$ and the sulfate group of SGG. However, upon Sr^{2+} addition, the relative intensity of the S=O asymmetric stretching mode decreased, indicating a relatively weak electrostatic interaction between Sr^{2+} and the SGG sulfate group.

The variation in electrostatic interaction strength can be due to many factors. The factor that probably contributes the most to this variation is the "charge concentration" of each ion. The Sr^{2+} ion is relatively large and bulky in comparison to Ca^{2+} and Mg^{2+} , and consequently, its charge is diffuse. In contrast, both Ca^{2+} and more so, Mg^{2+} are small "hard" ions with concentrated charge. Thus, with more concentrated charge, Ca^{2+} and Mg^{2+} are able to electrostatically interact with greater strength. Another factor to consider is the intermolecular distance between neighbouring lipid head groups, which is in the range of 5 Å. A Na^+ ion with one H_2O molecule is 5 Å in diameter (Stryer, 1988). An ion that is highly solvated or large and bulky would have a difficult time squeezing between neighbouring head groups due to opposing factors such as steric hindrance and the overwhelming resistance of the head groups to separate as a result of intermolecular hydrogen bonding. Finally, the electronegativity of an ion affects its electrostatic interactions. The electronegativity of an ion is a measure of its ability to attract electrons; the higher the electronegativity, the higher its ability to attract electrons. The electronegativity of Mg^{2+} is greater than Ca^{2+} which, in turn, has a greater electronegativity than Sr^{2+} . Therefore, several factors affect the electrostatic interaction

strength between $\text{Ca}^{2+}/\text{Mg}^{2+}/\text{Sr}^{2+}$ and the sulfate moiety of SGG. Unfortunately, this study cannot answer the question of which factor has the greatest influence. However, we did attempt to remove the factor of hydration, and performed the study using solid SGG.

The S=O asymmetric stretching mode of solid SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} was analyzed and several interesting findings were made (Figure 3.20). Firstly, the intensity of the low frequency band varied considerably, depending upon the cation. Upon Ca^{2+} addition, the intensity of the low frequency band increased significantly. Moreover, the peak absorption frequency of the high frequency band decreased from 1222 to 1218 cm^{-1} . The decrease in peak absorption frequency combined with the increase in absorption intensity indicated that Ca^{2+} bound tightly to the sulfate moiety. Removal of water, or rather desolvation of the ion allowed the Ca^{2+} ion to come in closer proximity with the negatively charged sulfate group and form a tight ionic bond. In contrast, Mg^{2+} only caused a small increase in intensity of the low frequency band with no change occurring to the high frequency band. This result suggests that Mg^{2+} only formed a weak electrostatic interaction with the sulfate group. However, due to the divalent nature of Mg^{2+} it likely crosslinked the sulfate groups of neighbouring lipid molecules. Given the small size and concentrated charge of the Mg^{2+} ion, it was likely unable to come in close proximity with either neighbouring sulfate groups. In contrast, the large size of the Ca^{2+} , or rather the more diffuse charge of Ca^{2+} , allowed it to come in closer proximity of the sulfate groups when crosslinking the neighbouring molecules and bind tightly to them. In the case of Sr^{2+} , the intensity of the low frequency

component is significantly lowered indicating a very weak electrostatic interaction with the sulfate moiety. Desolvation of the Sr^{2+} ion clearly fails to enhance its interaction with the sulfate moiety. In the solid state, intermolecular hydrogen bonding is enhanced and as a consequence, the distance between neighbouring molecules is likely decreased. Possibly, due to steric hindrance this decreased intermolecular distance prevents the bulky Sr^{2+} ion from crosslinking neighbouring lipid molecules.

Next, the S-O stretching mode of hydrated SGG was analyzed to provide further evidence of any interaction between the divalent cations and the negatively charged sulfate group (Figure 3.21). The increase in peak absorption frequency of this mode upon addition of all three divalent cations reflects an increase in the S-O bond strength. This increase in bond strength is likely due to resonance stabilization of the sulfate group, upon divalent cation interaction. Rather, the S-O bond has become a hybrid of a S=O bond and a S-O bond due to the electrostatic interaction with the divalent cations.

Finally, the C-O stretching mode of the ester-linked sulfate group was analyzed to determine whether the overall conformation of the sulfate was affected by the divalent cation interaction (Figure 3.22). The slight increase in bandwidth indicates that the conformation of the sulfate moiety became slightly more disordered upon divalent cation interaction.

4.3.2 The Interfacial Region (Ester Group)

The interfacial region of the hydrated SGG lipid molecule would likely be involved in hydrogen bonding with itself, neighbouring SGG lipid molecules, and H_2O .

Any changes in hydrogen bonding of this region due to divalent cation interaction with the head group would be reflected in the ester C=O stretching mode. The C=O stretching mode of SGG-Na⁺, SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ absorbed as a broad band comprised of a high-frequency and low-frequency band which were assigned to the free carbonyl group and the hydrogen bonded C=O, respectively (Figure 3.23). Comparison of the spectra of all four samples revealed different values for the intensity of the low frequency component. This indicates that the divalent cation interaction with the SO₃⁻ group of the lipid molecule affected the hydrogen bonding of the ester group differently, depending upon the cation. Of all the SGG-cation complexes, the low frequency component of SGG-Na⁺ (1715 cm⁻¹) absorbed with the highest intensity, indicating that the greatest proportion of the C=O groups in the sample was hydrogen bonded. The SGG-Mg²⁺ spectrum had a slightly lower intensity of absorption at this low frequency component (1715 cm⁻¹) as compared to the intensity of SGG-Na⁺, indicating a small decrease of the hydrogen-bonded C=O group population of SGG-Mg²⁺. In contrast to SGG-Na⁺ and SGG-Mg²⁺, SGG-Sr²⁺ absorbed at a much lower intensity at this low frequency band, followed by SGG-Ca²⁺. This indicates that a smaller proportion of the C=O group of SGG-Sr²⁺ and SGG-Ca²⁺ was participating in hydrogen bonding. The identity of the 1700 cm⁻¹ band of SGG-Na⁺ is unknown but is possibly an effect of interdigitation.

The effect of pressure on the hydrogen bonded C=O moieties was monitored in figure 3.24. Both mechanical and electronic factors influence the C=O stretching frequency where the C=O group is hydrogen bonded to a hydroxyl group (on the sugar

head group). When the carbonyl oxygen is hydrogen bonded to a neighbouring OH group, its free vibration becomes mechanically restricted and as a consequence, the C=O stretching frequency decreases (Mushayakarara, Wong, and Mantsch, 1986). In contrast, hydrogen bonding of the C=O group causes the lone pair electron density of the oxygen atom to migrate towards the hydrogen atom on the OH group. This partial electron withdrawal leads to a tighter C=O bond, a larger C=O stretching force constant, and as a consequence, an increase in the C=O stretching frequency (Mushayakarara et al., 1986). At ambient pressure, the mechanical effect outweighs the electronic effect, and thus upon hydrogen bonding the C=O stretching frequency decreases 20 to 30 cm^{-1} .

The barotropic behaviour of the SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} low-frequency component (hydrogen bonded C=O) was compared in figure 3.24. For SGG- Ca^{2+} and SGG- Mg^{2+} , as the pressure increased, the SGG C=O stretching frequency of the hydrogen bonded C=O group decreased for the entire pressure range. This indicated that throughout the pressure range studied, the mechanical effect is stronger than the electronic effect. In contrast, for SGG- Na^+ and SGG- Sr^{2+} , as the pressure increased, the C=O stretching frequency of the hydrogen bonded C=O group decreased slightly at first (up to ~ 8 kbar), but then remained constant at higher pressures. This implies that at pressures below 8 kbar the mechanical effect is stronger than the electronic effect. However, at pressures above 8 kbar, the pressure enhancement of the electronic effect and that of the mechanical effect were similar and offset one another.

The higher frequency of the SGG-Na⁺ low frequency band (1717 cm⁻¹ at ambient pressure) suggest that it is not as strongly hydrogen bonded as the C=O groups of SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ (1714-1715 cm⁻¹ at ambient pressure) (Figure 3.24). Moreover, the slightly lower peak absorption frequencies of the SGG-Ca²⁺ and SGG-Sr²⁺ low-frequency bands imply that their C=O groups are more strongly hydrogen bonded and more mechanically restricted than those of SGG-Mg²⁺. This variation in hydrogen bonding strength could be a result of the variation in hydrogen bonding donors (H₂O, intra- vs. intermolecular hydrogen bonding) and/or mechanical restraint due to the variation in intermolecular distance between lipid molecules.

Referring back to figure 3.23, the lower frequency of the SGG-Na⁺ high-frequency component (1735 cm⁻¹) indicated that the free vibration of the carbonyl oxygen atom is mechanically restricted, and as a consequence, its absorption frequency is reduced in comparison to SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ (1738 cm⁻¹). This suggest that the environment of the SGG-Na⁺ C=O moiety is slightly different than that of SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺.

4.3.3 The Hydrocarbon Chain Structure

The hydrocarbon chain dynamics of SGG-Na⁺, SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ were compared by analyzing several absorption modes: the CH₂ scissoring (δ CH₂) and rocking modes (γ CH₂), and the CH₃ bending mode. In addition, two spectroscopic parameters were measured to facilitate identification of bilayer interdigitation: the peak height intensity ration (R) of δ' CH₂ and δ CH₂ and the height intensity ratio (R') of γ' CH₂

and its corresponding "valley" component.

Comparison of the stacked contour plots of the CH_2 scissoring mode of SGG- Na^+ (Figure 3.25A), SGG- Ca^{2+} (Figure 3.25B), SGG- Mg^{2+} (Figure 3.26A), and SGG- Sr^{2+} (Figure 3.26B) revealed that the SGG- Na^+ spectra differed in its barotropic behaviour. The pronounced "valley" defined by the two component bands of the SGG- Na^+ CH_2 scissoring mode differs in its appearance in comparison to the "valley" formed by the correlation field splitting of the SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} CH_2 scissoring modes, and suggests interdigitation of the SGG- Na^+ bilayer. Siminovitch, Wong, and Mantsch (1987) have demonstrated that the qualitative features of bandshapes, when displayed in a stacked contour plot, can be used to distinguish interdigitated bilayer systems. In fully interdigitated lipid bilayers, the interchain interactions are significantly enhanced and are reflected in the CH_2 scissoring and rocking modes. The broad "valley", or rather the large magnitude of splitting, produced by the correlation field splitting of the SGG- Na^+ CH_2 scissoring mode signifies the more rapid enhancement of interchain interactions upon compression of an interdigitated bilayer. The presence of only a single, narrow CH_2 scissoring band at ambient pressure for all four lipid samples, regardless of whether the SGG hydrocarbon chains were interdigitated or not, indicated that the chains were fully extended and that there were still significant chain reorientational fluctuations at ambient pressure. The inability to discriminate between interdigitated and non-interdigitated systems at ambient pressure stresses the fact that only under increasing pressure can the differences in interchain packing and dynamical structure be clearly reflected in the infrared bandshapes.

Additional evidence to support identification of SGG- Na^+ interdigitation is shown in figure 3.27, which displays the pressure dependence of the peak height ratio (R) of $\delta'\text{CH}_2$ and δCH_2 , the high- and low-frequency components of the correlation field splitting of the CH_2 scissoring mode. Analysis of this figure reveals both large and small scale fluctuations in the R parameter with increasing pressure. The small scale fluctuations (± 0.02) are inherent in the calculations and due to deviations in the height measurements. The large scale fluctuations, particularly evident for SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} , appear as sudden increases in the R parameter followed by plateaus of no change. These large scale fluctuations are most likely due to pressure-induced structural phases transitions (Siminovitch, Wong, and Mantsch, 1987). The magnitude of the difference of R between SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} reflect subtle differences in interchain interactions and chain dynamics, and is small in comparison to the difference between non-interdigitated and interdigitated systems (Siminovitch et al., 1987).

Analysis of the R pressure profiles in figure 3.27 revealed the peak height ratios (R) in SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} are significantly higher than the corresponding ratio in the interdigitated SGG- Na^+ (Tables 3.9, 3.10, 3.11, and 3.12). Siminovitch et al. (1987) demonstrated that the peak height ratio in non-interdigitated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine is always greater than the corresponding ratio in interdigitated systems. Indeed, they proposed that the peak height ratio $R = I_{\delta'}/I_{\delta}$ of the CH_2 scissoring mode components as a useful, alternative method of determining chain interdigitation. Moreover, Siminovitch et al. (1987) predicted that the

relative intensities of the CH_2 rocking correlation field splitting components are sensitive to the effect of interdigitation on the dynamical structure of the hydrocarbon chains. However, they found quantitation of CH_2 rocking mode intensities difficult and unreliable due to the shoulders of the intense quartz 801 and 695 cm^{-1} bands and the variable baseline. In this study, we have introduced a new parameter, R' , in which quantitation is reliable and relatively simple, if done properly.

The stacked contour plots of the infrared spectra of hydrated SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in the frequency range 675 to 840 cm^{-1} are shown in figures 3.29, 3.30, 3.31, and 3.32 respectively. The CH_2 rocking mode absorbs at $\sim 720 \text{ cm}^{-1}$ at ambient pressure, with its correlation field splitting component (arrow) absorbing at ~ 720 to $\sim 740 \text{ cm}^{-1}$ over increasing pressure. To maintain a stable baseline over increasing pressure, the spectra was plotted over a wide frequency range. Ideally, the frequency range plotted should border at frequencies where no vibrational modes absorb. Clearly, the frequency range in which the CH_2 rocking mode absorbs is quite crowded, and as a consequence, we had to plot a wide frequency range in order to satisfy the requirement of a stable baseline. Had the band heights been measured over a more narrow frequency range, the shifting absorptions that border the bands would have disrupted the baseline. By measuring band heights relative to a parameter that is variable rather than constant would have lead to inaccurate and distorted measurements.

Comparison of the stacked contour plots of the CH_2 rocking mode revealed a different barotropic behaviour of the SGG- Na^+ spectra (Figure 3.29) in comparison to SGG- Ca^{2+} (Figure 3.30), SGG- Mg^{2+} (Figure 3.31), and SGG- Sr^{2+} (Figure 3.32) spectra. The intensity of the SGG- Na^+ $\gamma'\text{CH}_2$ band increased more rapidly with increasing pressure (Figure 3.29, Table 3.13), producing a deep and wide "valley" between the two component bands. Due to the interfering shoulder of the quartz band that absorbs at 695 cm^{-1} and shifts in frequency with pressure, the

The intensity of the SGG-Na⁺ γ' CH₂ band increased more rapidly with increasing pressure (Figure 3.29, Table 3.13), producing a deep and wide "valley" between the two component bands. Due to the interfering shoulder of the 695 cm⁻¹ quartz band, the intensity of the γ CH₂ band could not be reliably quantitated. However, by quantitating the height of the "valley" between the two component bands, we have introduced a new spectroscopic parameter, R'. The height ratio, $R' = I_{\gamma'CH_2}/I_{\text{"valley"}}$ is a parameter which factors in, not only the height of the correlation field splitting component of the CH₂ scissoring mode, but also the influence of the γ CH₂ band has on the valley. Obviously, the "valley" height is a function of the two component heights and the magnitude of their splitting. Consequently, the "valley" height would be sensitive to the interchain interactions, and the height ratio, $R' = I_{\gamma'CH_2}/I_{\text{"valley"}}$ would be a useful parameter to reflect the differing barotropic behaviour of an interdigitated system's CH₂ rocking mode. It should be noted that the R' parameter is not intended to be used as a quantitative measurement of interdigitation but a measurement that reflects the different qualitative features of the CH₂ rocking mode. When plotted over increasing pressure the R' parameter shows that the qualitative features of a interdigitated and noninterdigitated CH₂ rocking mode display different trends. Indeed, the pressure profile of the SGG-Na⁺ R' parameter was significantly different compared to those of SGG-Ca²⁺, SGG-Mg²⁺, and SGG-Sr²⁺ (Figure 3.33). The rapid increase in the SGG-Na⁺ R' parameter reflects the production of a deep "valley" between the two component bands. The deep "valley" is produced from the greater magnitude of splitting of the SGG-Na⁺ CH₂ rocking mode. The extent of the correlation field splitting of the CH₂ rocking mode is interchain in origin, and Siminovitch et al. (1987) found that the greater magnitude of splitting in 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine lipid bilayers is a direct reflection of an interdigitated lamellar structure. It seems likely that this is the case for SGG. Evidently, in the presence of Na⁺, SGG bilayers were interdigitated, however, upon addition of the divalent

cations Ca^{2+} , Mg^{2+} , and Sr^{2+} , the SGG bilayers became non-interdigitated. Most likely, the divalent cations were crosslinking the SGG head groups and preventing interdigitation of the SGG lipid molecules.

The effect of the divalent cations on the hydrocarbon chain dynamics was determined by comparing the pressure dependence of the CH_2 scissoring mode frequencies of SGG- Na^+ , SGG- Ca^{2+} , SGG- Mg^{2+} , and SGG- Sr^{2+} in figure 3.28. The higher splitting pressure of SGG- Mg^{2+} (4.2 kbar) indicated that it induced the greatest chain disorder followed by SGG- Ca^{2+} (3.8 kbar). The lower splitting pressure of SGG- Sr^{2+} (3.5 kbar) reflected only a small increase in chain disorder compared to SGG- Na^+ (3.3 kbar). However, the value of the SGG- Sr^{2+} splitting pressure is questionable due to a jump in the pressure (from 2.5 to 3.5 kbar) just prior to the correlation field splitting. Consequently and unfortunately, the splitting pressure of SGG- Na^+ and SGG- Sr^{2+} cannot be accurately compared. Nonetheless, not only did all three divalent cations eliminate bilayer interdigitation, but both Ca^{2+} and Mg^{2+} induced greater hydrocarbon chain disorder.

Finally, the CH_3 bending mode of SGG was analyzed to characterize the differences between the interdigitated and non-interdigitated systems (Figure 3.34). Clearly, the interactions of the terminal methyl groups of the interdigitated SGG- Na^+ would be expected to differ from those of the non-interdigitated SGG bilayer. The lower peak absorption frequency of SGG- Na^+ suggested that the methyl group interactions are relatively weak compared to SGG- Ca^{2+} and SGG- Mg^{2+} , as would be expected in an interdigitated system. Interestingly, the peak absorption frequencies of SGG- Sr^{2+} were found to be intermediate between SGG- Na^+ and SGG- Ca^{2+} /SGG- Mg^{2+} . This difference in peak absorption frequency indicates that the methyl group interactions of SGG- Sr^{2+} are quite different from SGG- Mg^{2+} and SGG- Ca^{2+} . The results suggest that the interchain packing of SGG- Sr^{2+} differs from that of SGG- Ca^{2+} and SGG- Mg^{2+} . Unfortunately, the interchain packing cannot be deduced from the correlation field splitting of the CH_2 scissoring

or rocking modes. Although it is known that correlation field splitting is only observed when the hydrocarbon chains are packed in a monoclinic or orthorhombic crystal lattice (Snyder, 1961), the precise nature of the interchain packing in SGG when interacting with various types of divalent cations cannot be determined without X-ray diffraction measurements.

In summary, we have shown that hydrated SGG- Na^+ is interdigitated and that the divalent cations Ca^{2+} , Mg^{2+} , and Sr^{2+} electrostatically interact with the negatively charged sulfate group, likely by crosslinking neighbouring lipid molecules, and thus preventing the SGG molecules from interdigitating. Each divalent cation apparently has a different affinity for the sulfate group and electrostatically interacts with differing strengths. The spectroscopic evidence indicates that Ca^{2+} interacts with the greatest strength, followed by Mg^{2+} , then Sr^{2+} . Moreover, each divalent cation increases the hydrocarbon chain disorder to differing degrees; Mg^{2+} induces the greatest disorder, followed by Ca^{2+} , then Sr^{2+} . The hydrogen bonding of the interfacial region is also affected to different degrees by the divalent cations. While it appears that a high proportion of the SGG- Mg^{2+} C=O groups are hydrogen bonded, the SGG- Ca^{2+} /SGG- Sr^{2+} C=O groups are more strongly hydrogen bonded, yet fewer in number. Finally, it appears that SGG- Sr^{2+} differs from SGG- Ca^{2+} /SGG- Mg^{2+} in its hydrocarbon chain packing. Presumably, these vast differences in the divalent cation effects on the SGG lipid structure and dynamics are inherent in the properties of the divalent cations. These three divalent cations, Ca^{2+} , Mg^{2+} , and Sr^{2+} differ in their size, charge distribution, solvation, and electronegativity. Clearly, these physical properties influence the effects

that each divalent cation has on the structure and dynamics of the sulfoglycolipids. Perhaps this provides a partial explanation as to why differences in their physiological effects were observed for these divalent cations (Stack and Fraser, 1989; Yanagimachi and Usui, 1974; Rogers and Yanagimachi, 1986; Roldan and Harrison, 1989). It is not just a simple matter of divalency acting upon a charge. Rather, several other factors are influencing the physiological effects of a cation.

4.4 Significance of Research Findings

The fluid mosaic model of membrane permits membrane proteins to function in a dynamic manner within the lipid bilayer (Singer and Nicholson, 1972). However, this model gives the false impression that the only role of membrane lipids is to provide a favourable environment of proper fluidity. If this were the case, it is unlikely that there would be such a variety of lipids, some of which are membrane-, tissue-, or species-specific. If control of fluidity was the sole function of lipids, it could be simply achieved by variations in chain length and degree of chain unsaturation (Boggs, 1980). This study examined the interaction of divalent cations with two structurally related sulfoglycolipids, SGC and SGG. The purpose of this study was to characterize the molecular forces of these sulfoglycolipids such as those arising from binding of divalent cations, which control their behaviour and dynamics. The information obtained could lead to a better understanding of their individual roles in plasma membrane.

While the importance of Ca^{2+} in mediating sperm membrane interactions during

fertilization-related events has been well established (Yanagimachi, 1978, 1982; Saling et al., 1978) and the relationship between Ca^{2+} , plasma membrane, and motility have been described (Babcock et al., 1978, 1979), very little is known about the direct interaction of Ca^{2+} with the sperm plasma membrane. This study has shown that Ca^{2+} interacts with two sperm plasma membrane sulfoglycolipids, SGC and SGG, in model membrane systems. This interaction results in an increase in SGC and SGG lipid dynamics and the removal of SGG bilayer interdigitation. Although it is not yet possible to draw conclusions with respect to the physiological significance of these results in the intact sperm, these findings merit further consideration. This model study may prove relevant in future studies on sperm plasma membrane and intact sperm.

Like all glycolipids, SGC and SGG are likely present on the outer leaflet of the plasma membrane bilayer (Hakomori, 1981). It is plausible that SGC and SGG may be anionic sites on the cell surface that serve as "traps" of Ca^{2+} and other cations. Indeed, it has been shown that Ca^{2+} is present on the sperm surface, although its binding molecule(s) is unknown (Ruknudin, 1989).

The influx of Ca^{2+} into sperm coincides with many fertilization-related events including capacitation and the acrosome reaction (Babcock et al., 1978, 1979; Florman et al., 1992, Cox et al., 1991). Peterson et al. (1979), by using the patch-clamp technique, have shown that plasma membrane vesicles of boar sperm bind Ca^{2+} with high affinity and have suggested that the Ca^{2+} binding properties of the plasma membrane influence Ca^{2+} transport across the membrane. Moreover, it has been established that channels that conduct divalent cations exist in mammalian sperm plasma membrane.

Studies to determine the type of Ca^{2+} channels in mammalian sperm plasma membrane have been inconclusive to date, although several studies have provided indirect evidence of voltage-gated Ca^{2+} channels (Babcock and Pfeiffer, 1987; Florman et al., 1992). SGC and SGG may influence Ca^{2+} transport across the sperm plasma membrane by trapping followed by releasing Ca^{2+} prior to the ion influx through Ca^{2+} channels. Moreover, the increase in SGC and SGG lipid dynamics upon Ca^{2+} binding (Figures 3.16, 3.17, 3.25, and 3.27) may be one of the regulatory functions that influences the activity of Ca^{2+} channels. Indeed, Benjamins and Dyer (1990) have reported that exposure of cultured oligodendrocytes to anti-GC and anti-SGC antibodies induces the opening of Ca^{2+} channels, suggesting that the glycolipids may influence that activity of Ca^{2+} channels.

Both capacitation and the acrosome reaction involve significant structural changes to the sperm plasma membrane (Yanagimachi, 1988). The most significant modifications that accompany capacitation include: 1) Changes of lipid components such as a decrease in cholesterol to phospholipid ratio, and an increase in lysophospholipid levels due to an increased activity of sperm plasma membrane phospholipase A2. These changes result in an increase in membrane fluidity and porosity; 2) An influx of Ca^{2+} into the sperm due to the increase in membrane porosity; 3) Changes to protein and sugar residues with exposure of zona receptors on the sperm surface; 4) An increase in adenylate cyclase activity; 5) A conversion of proacrosin to acrosin; and 6) An acquisition of hyperactivated motility patterns (Saling, 1989; Go and Wolf, 1983; Wolf et al., 1986; Koehler, 1981; Fraser, 1990; Langlais and Roberts, 1985; Parrish and Polakoski, 1979; Burkman, 1990). Only capacitated sperm, and not uncapacitated sperm, can bind to the

zona pellucida and the event is Ca^{2+} -dependent (Saling et al., 1978). The agonist, zona pellucida, then causes a massive Ca^{2+} influx into the sperm, the event that is operated through the G_i protein mechanism (Kopf and Gerton, 1990; Florman and First, 1988). Ca_i^{2+} triggers the signal transduction cascade, the details of which are still unknown. However, this ends with the exocytotic event, the acrosome reaction, which occurs in a stepwise manner. It starts with the fusion of the plasma membrane with the outer acrosomal membrane resulting in sperm plasma membrane vesiculation. The acrosomal contents are then released until the sperm inner acrosomal membrane is exposed on the sperm anterior head surface. While it is well documented in the literature that changes in the levels of cholesterol, phospholipids, and lysophospholipids may play important roles in enhancing the membrane fluidity and membrane fusion events (Wolf et al., 1986; Yanagimachi, 1988), our findings that Ca^{2+} binds to SGG/SGC increases the lipid dynamics warrants consideration of similar roles of these sulfoglycolipids on the sperm membrane. Especially for SGG, the sulfoglycolipid is found at a high level, i.e. 10^8 molecules/sperm and at least half of it is accumulated in the membrane overlying the acrosome (Gadella et al., 1991, 1992; Tanphaichitr et al., 1993; Vos, Lopes-Cardozo, and Gadella, 1993).

The differing affinities of Ca^{2+} , Mg^{2+} , and Sr^{2+} for SGG and the corresponding variation in lipid dynamics and packing effects may provide some explanation as to why Mg^{2+} and Sr^{2+} cannot always mimic Ca^{2+} in certain fertilization events such as capacitation and the acrosome reaction (Rogers and Yanagimachi, 1976; Stock and Fraser, 1989). Roldan and Harrison (1989) have determined using a Ca^{2+} /ionophore-

induction system that there are three separate Ca^{2+} -dependent events that can be distinguished during the build-up of exocytosis. The first event is the phosphoinositide breakdown, in which Ca^{2+} is an absolute requirement. The two subsequent Ca^{2+} -dependent events have yet to be elucidated; however, Sr^{2+} can effectively substitute for Ca^{2+} in one of these two. It is speculated that one of these two events may be Ca^{2+} -dependent membrane fusion. It is likely that the membrane structural changes that occur during these events are very specific and even slight changes to the lipid dynamics can prevent the completion of the acrosome reaction. It is possible that the lipid dynamics of SGG- Ca^{2+} (or SGG- Sr^{2+}) facilitate the membrane structural changes necessary for the acrosome reaction. On the other hand, the lipid dynamics of SGG- Na^+ , SGG- Mg^{2+} , and SGG- Sr^{2+} may prevent or counteract these required membrane structural changes.

The ability of SGG- Na^+ bilayers to interdigitate in model membranes may be nonphysiological. Interdigitated bilayers have not yet been demonstrated to occur *in vivo*, although bilayer interdigitation has been suggested to occur during the structural transitions involved in membrane fusion. Since SGG is likely on the outer leaflet of the sperm plasma membrane (Hakomori, 1981), it would have to interdigitate with other hydrocarbon chain containing lipids. The candidates of these lipids would be those that are fusogenic, including free fatty acids, lysophospholipids, and plasmalogen (containing an ether linkage at the *sn*-1 position) (Ruocco et al., 1985; Slater and Huang, 1988; Papahadjopoulos et al., 1990; Boggs, 1987; Cullis and de Kruijff, 1978). While interdigitation of the lipids between the two bilayer leaflets may cause an initial disorder to the membrane, the tight interaction (i.e., high-density packing) between the

hydrocarbon chains of the outer and inner leaflets of the bilayer would influence not only the fluidity of the interdigitated domain, but the ability of the lipids to form laterally segregated domains within the bilayer plane (Slater and Huang, 1988). As shown for SGG, interdigitation of SGG was abolished after SGG-bound Na^+ was replaced by divalent cation (Figure 3.25, 3.26, 3.27, 3.28, and 3.33). Concurrently, it appears that divalent cations such as Ca^{2+} could increase the SGG lipid dynamics significantly as compared to the dynamics of SGG- Na^+ . Although Ca^{2+} is present throughout the female reproductive tract (Yanagimachi, 1988), the amount of Ca^{2+} bound to SGG on the sperm plasma membrane may vary depending upon the following; 1) The relative levels of other cations; 2) The presence of Ca^{2+} binding proteins (i.e. caltrin, calmodulin, prothrombin, etc.) (Coronel and Lardy, 1992; Lardy et al., 1988; Papahadjopoulos et al., 1990; Stryer, 1988); 3) Masking of SGG by other macromolecules such as SLIP1; and 4) Segregation of SGG from SLIP1 (Tanphaichitr et al., 1992, 1993). Presumably, all of these factors would determine the dynamics of SGG on the sperm plasma membrane.

The release of arylsulfatase-A during the acrosome reaction results in the desulfation of SGC and SGG to form their respective parental compounds, GC and GG. Removal of the negatively charged sulfate group reduces the number of Ca^{2+} "traps" on the sperm surface, which might then enhance Ca^{2+} influx into the sperm. Moreover, the reaction products, GC and GG can aggregate via enhanced intermolecular hydrogen bonding which would promote membrane stability (Figures 3.12 and 3.13) (Sen et al., 1983). On the other hand, GG has fusogenic properties due to its ether linkage and may play a role during the process of the acrosome reaction or the sperm-egg plasma

membrane fusion. Further, GG may be converted by phospholipase to lysoGG, which is similar in structure to lysoGC (i.e., galactosylsphingosine), a lipid thought to play a role in signal transduction (Hannun and Bell, 1989).

The binding of sperm to the zona pellucida surrounding the egg requires extracellular Ca^{2+} (Saling et al., 1978). It is possible that SGG is involved in sperm-zona pellucida binding given its significance in fertility and the fact that its specific binding protein (SLIP1) is involved in sperm-zona pellucida binding (Tsuji et al., 1992; Lingwood et al., 1990; Tanphaichitr et al., 1992, 1993). Moreover, the increase in SGG lipid dynamics upon Ca^{2+} binding and possibly SLIP1-SGG binding, may facilitate the translocation of SLIP1 to the apical ridge of the sperm head, the initial zona pellucida binding site (Moase and Tanphaichitr, 1992). Future studies on SLIP1 interactions with SGG/ Ca^{2+} model membranes as well as on intact sperm will hopefully elucidate the interactions and roles of SLIP1/SGG/ Ca^{2+} with respect to sperm-zona pellucida binding.

With respect to the roles of SGC in immune recognition and toxin receptor, Ca^{2+} involvement has not yet been established (Karlsson, 1989, 1991). However, if the binding of Ca^{2+} to SGC on the cell surface is necessary for these events, this interaction could enhance lipid fluidity (as our results have demonstrated in figures 3.16, 3.17, 3.25, and 3.27) and subsequently induce changes to lipid permeability, the lateral segregation of lipids and proteins, and the activity of plasma membrane proteins. Therefore, if SGC and SGG are cell surface receptors (of other cells, antibodies, toxins, hormones, etc.) which transmit or amplify signal to other membrane components, the lipid dynamic effects of Ca^{2+} should be considered as a factor in future investigation.

Although the lipid dynamic effects of Ca^{2+} binding to SGG/SGC may not be the sole factor in the changes of sperm membranes during fertilization, this study has helped us determine the molecular forces that control the behaviour and dynamics of SGC and SGG, and has helped us understand and speculate about their individual roles in plasma membrane. The results of this model membrane study can serve as ground work for future studies of SGG/SGC on the sperm plasma membrane or intact sperm, especially on their functions on the sperm plasma membrane as well as their interactions with other macromolecules such as SLIP1 or the zona pellucida.

APPENDIX ONE

THEORY OF INFRARED SPECTROSCOPY

A1.1 Background Theory on Infrared Dispersion Spectroscopy

There are several types of electromagnetic radiation, including visible light, ultraviolet, infrared, x-ray, etc. Terms used to describe light include wavelength and frequency. The wavelength, λ , is the peak-to-peak distance between waves. The frequency, ν , is the number of wave oscillations made each second. Each type of radiation is part of the broad electromagnetic spectrum that ranges from gamma rays, which have wavelengths in the magnitude of an Angström unit, to radio waves, which have wavelengths in the magnitude of meters to kilometers (Harris, 1987). All types of electromagnetic radiation travel at the same velocity of 3×10^{10} cm/second in vacuum, but differ in their wavelength, and consequently their frequency. Frequency is inversely related to the wavelength:

Equation A1

$$\nu = c/\lambda$$

ν = frequency, in sec^{-1}

λ = wavelength, in cm

c = velocity, 3×10^{10} cm/sec

Therefore, the longer the wavelength, the lower the frequency.

With respect to energy, light can be thought of as particles called photons (Harris, 1987). Each photon contains energy. According to Planck's constant, the amount of energy, E , in a photon of radiation is directly related to its frequency:

Equation A2

$$E = h \nu$$

E = energy, in ergs
h = Planck's constant,
 $= 6.5 \times 10^{-27}$ erg-sec

By combining equations 1 and 2:

Equation A3

$$E = hc/\lambda = hc\tilde{\nu}$$

$\tilde{\nu}$ = wavenumber, cm⁻¹

$$\tilde{\nu} = 1/\lambda$$

Therefore, the energy in a photon is inversely proportional to its wavelength and directly proportional to its wavenumber. The major regions of the electromagnetic spectrum are shown in figure A1.1, which shows the corresponding wavelengths, frequencies, and energies.

When a beam of electromagnetic radiation is directed through a molecule, the molecule can either absorb or transmit the radiation, depending upon its structure (Harris, 1987). When a molecule absorbs electromagnetic radiation, the energy of the molecule is increased and the molecule is promoted to an excited state. If a molecule transmits electromagnetic radiation, it emits a photon and its energy is lowered. A schematic representation of light absorption and emission is shown in Figure A1.2. A molecule's lowest energy state is called the ground state (Harris, 1987).

The absorption of light energy by a molecule may result in increased vibration or rotation of its atoms, or may cause its electrons to rise to higher energy levels. The frequency of electromagnetic radiation that a molecule can absorb depends upon the permitted changes in vibrations, rotations, or electronic states allowed by the structure of the molecule (Harris, 1987).

Figure A1.1 - The electromagnetic spectrum (from Harris, 1987, p497).

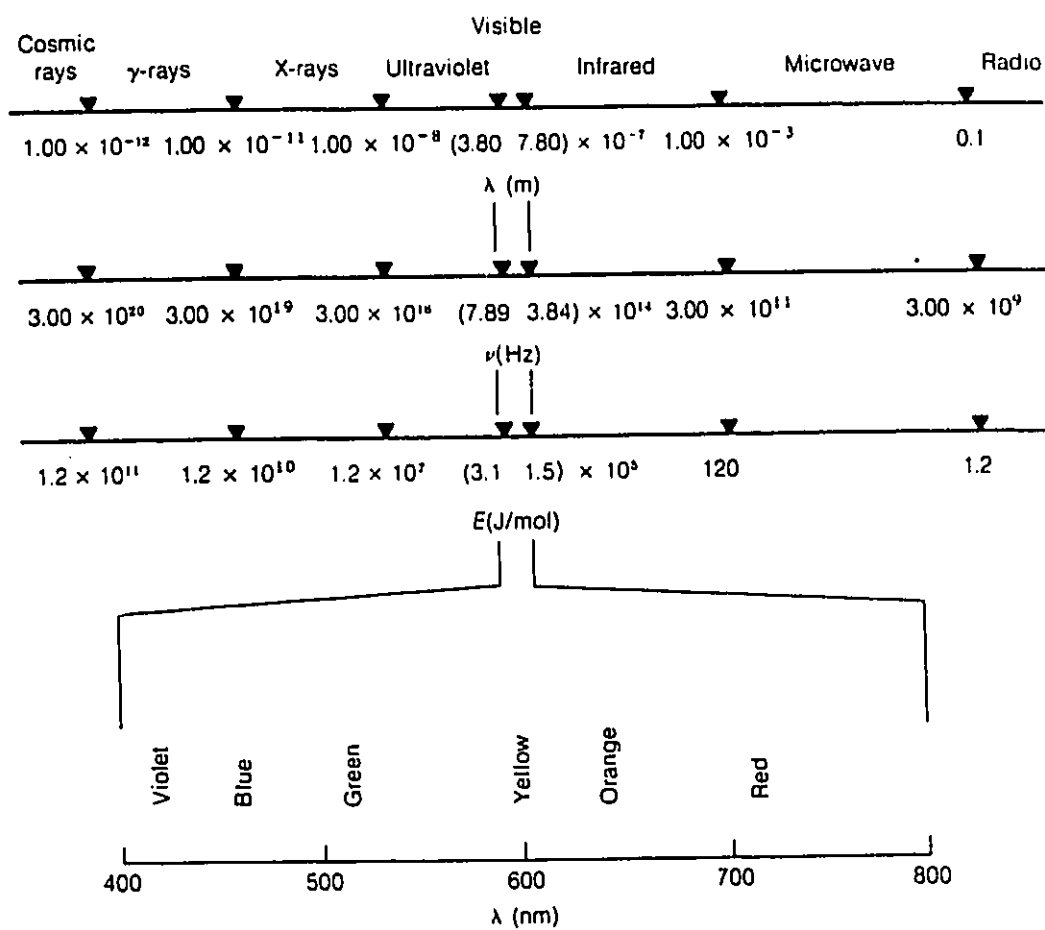


Figure A1.1

Figure A1.2 - Schematic diagram of light absorption and emission by a molecule (from Harris, 1987, p497).

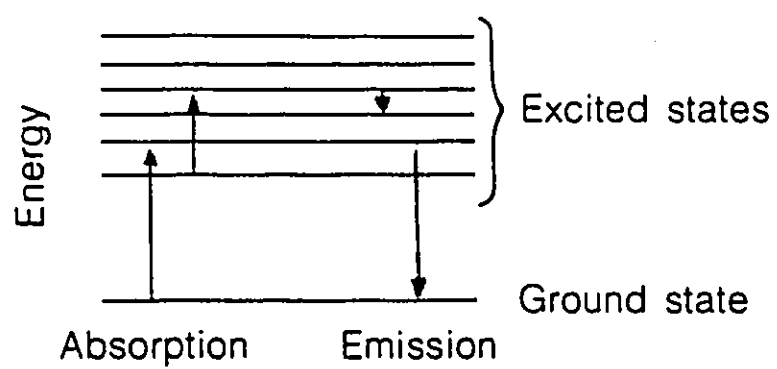


Figure A1.2

A molecule is always vibrating and its bonds are continually stretching and bending with respect to one another. When a molecule absorbs electromagnetic radiation in the infrared region of the spectrum, changes in the molecular vibrations result (Colthup, Daly, and Wiberly, 1975). Consider a molecule with two atoms of masses m_1 and m_2 . When a vibration occurs along its molecular axis, the molecule can be compared to a simple harmonic oscillator with a parabolic energy function (Figures A1.3 and A1.4A). The frequency of vibration is given by:

Equation A4
$$= \frac{1}{2\pi} \left[k \left(\frac{1}{m_1} + \frac{1}{m_2} \right) \right]^{1/2} \quad k = \text{Hook's law force constant}$$

However, a diatomic molecule differs from a true harmonic oscillator. It differs in two ways; 1) Vibration on an atomic scale is governed by quantum mechanic principles. It can only occur at energies E , where:

Equation A5
$$E = (n + 1/2)h\nu \quad n = 0, 1, 2, \dots$$

$$\nu = \text{frequency defined in equation A4}$$

2) The energy function of a diatomic molecule is not exactly parabolic, at higher energies the equilibrium between attractive and repulsive forces results in an asymmetric curve, causing an unequal separation of the upper energy levels (Figure A1.4B) (Warren, 1987).

Figure A1.3 - Schematic diagram of a simple harmonic oscillation consisting of two masses, m_1 and m_2 (from Warren, 1987, p164).

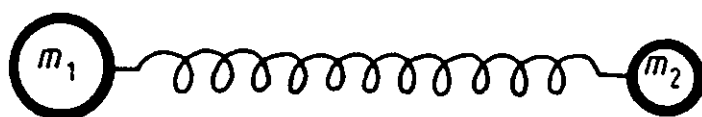


Figure A1.3

Figure A1.4 - (A) Energy of a simple harmonic oscillator as a function of the distance between the two masses.
(B) Energy of a diatomic molecule as a function of atomic separation (from Warren, 1987, p164).

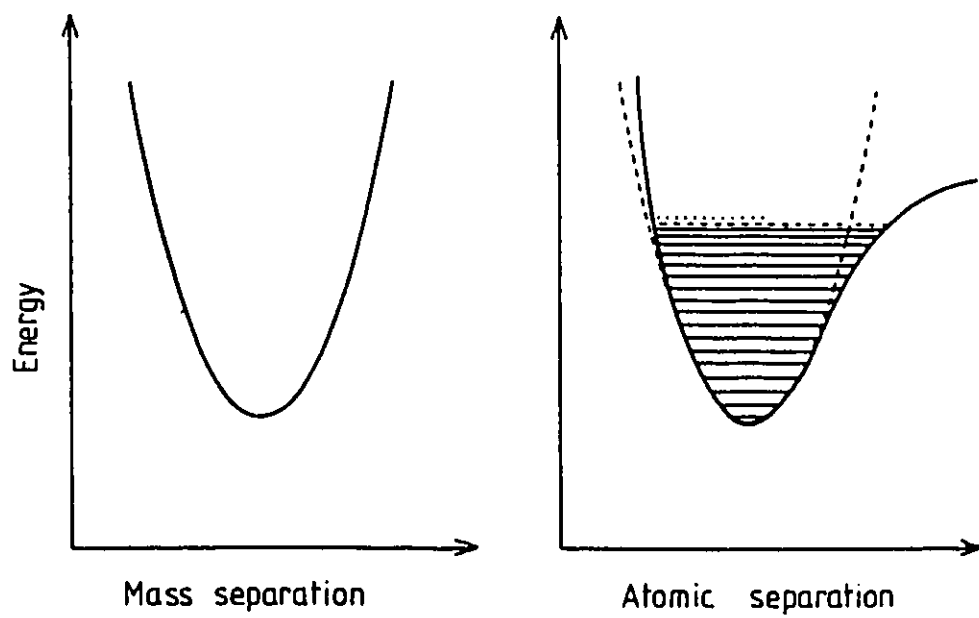


Figure A1.4

In complex molecules, several types or modes of vibration are possible, depending upon the chemical bonding of the molecule and the three-dimensional molecular geometry. A non-linear molecule with n atoms has $3n$ degrees of freedom (Colthup et al., 1975). Three degrees of freedom correspond to translation of the entire molecule in the x , y , or z directions. Another 3 degrees of freedom correspond to rotation about the three axes of the molecule. Finally, the remaining $3n-6$ degrees of motion correspond to vibrational motions, with each motion having a characteristic fundamental band frequency. However, certain vibrational motions do not allow absorption at their characteristic band frequency (Cross, 1964; Colthup et al., 1975). Absorption occurs only when a periodic change in the dipole moment is produced by the photon's electric field. Generally, if a molecule in its equilibrium configuration has a center of symmetry, a molecular vibration should not cause any change in the dipole moment and as a consequence a forced dipole moment oscillation cannot activate the vibration (Colthup et al., 1975). Therefore, total symmetry about a bond will eliminate the absorption of certain fundamental band frequencies.

There are two distinct types of bond vibrational modes: stretching and bending (deformation) vibrations (Cross, 1964). A stretching vibration consists of the periodic stretchings of the bond A-B along the axis of the bond. A bending vibration of the bond A-B is a displacement that occurs at right angles to the bond axis. Several different vibrational modes have been characterized and are schematically represented in figure A1.5 for both AX_2 and AX_3 groups.

Figure A1.5 - Schematic representation of the different types of stretching and bending vibrations for AX_2 and AX_3 groups (from Cross, 1964, p4).

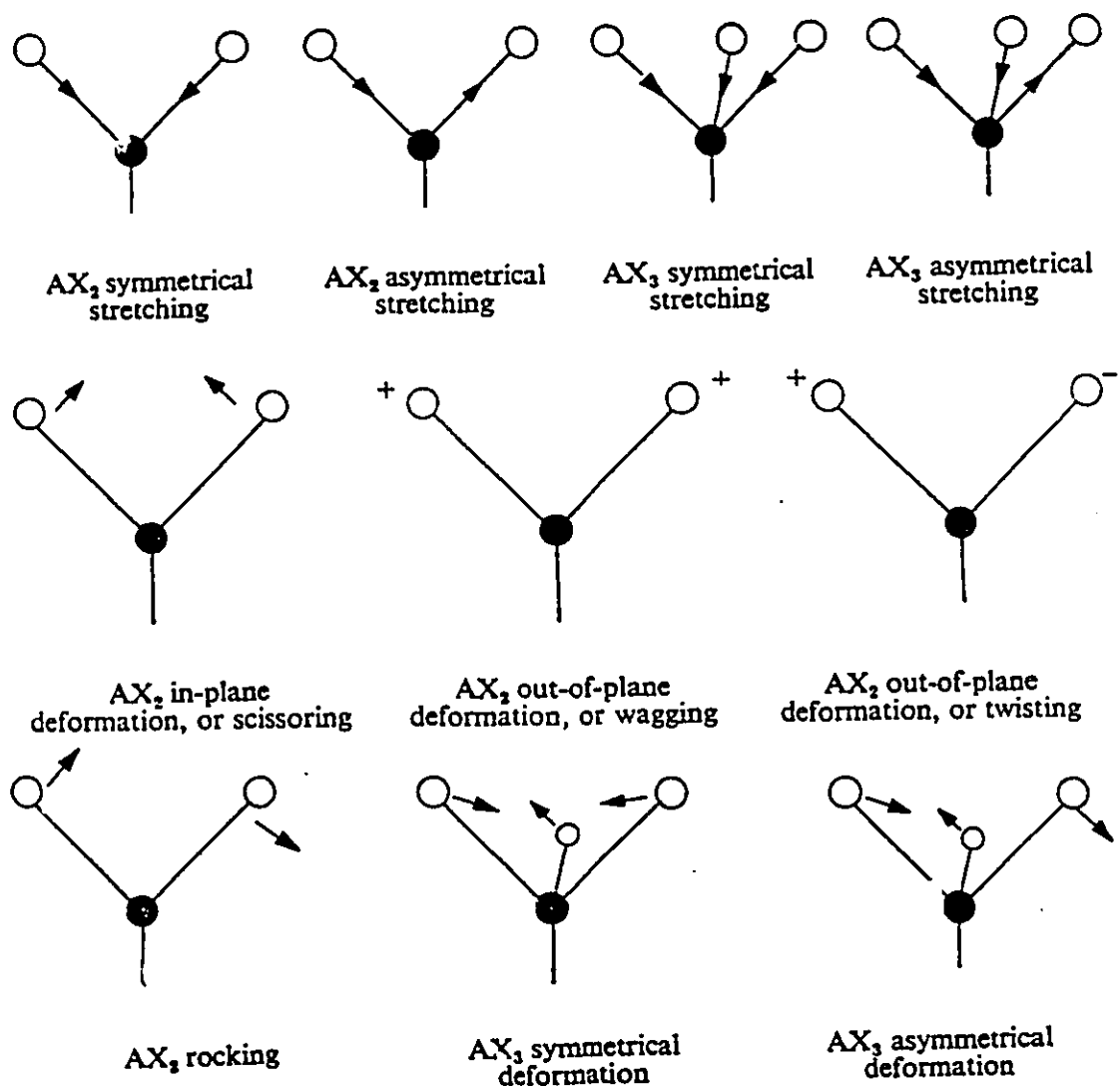


Figure A1.5

Different symbols have been designated by spectroscopists to differentiate between the various vibrational modes: ν is used for stretching vibration frequencies, δ for frequencies of deformations involving bond-angle changes, τ for twisting vibration frequencies, and δ' for out-of-plane deformation or wagging mode frequencies (Cross, 1964).

A1.2 Fourier-Transform Infrared (FTIR) Spectroscopy

The development of Fourier-transform techniques has allowed for the simultaneous recording of the entire infrared spectrum. By using a Michelson interferometer, a device that contains two orthogonal plane mirrors and an inclined semi-reflecting beam splitter, sinusoidal oscillations in the detected infrared signal are produced (Warren, 1987). The beam splitter splits the infrared signal. This reflects off the two orthogonal plane mirrors, recombines at the beam splitter, and interferes to produce maximal output when the two beams are in phase and zero output when they are 180° out of phase. By moving one of the mirrors at a constant velocity in a direction perpendicular to its plane, an alternating constructive and destructive interference results, producing a sinusoidal oscillation in the output. The oscillation frequency is proportional to the frequency of incident radiation. Placement of a sample between the infrared radiation source and the interferometer and detector produces a signal that is proportional to the Fourier transform of the absorption spectrum, which is subsequently retransformed using a computer. A large optical throughput is produced due to the absence of slits and by using signal averaging, the signal-to-noise ratio is much higher than that of a grating

dispersion spectrometer (Warren, 1987). The Fourier-transform infrared spectrophotometer is an extremely sophisticated instrument with several advantages including speed, frequency accuracy, more efficient use of radiation, improved signal-to-noise ratio, and built in data-handling capabilities (Harris, 1987). The superiority of FT-IR has led to the rapid displacement of traditional dispersion instruments.

A1.3 Fourier Analysis of Infrared Data

Fourier analysis of an infrared absorption spectrum is a practical approach used to extract more information from the infrared spectra of a sample. Fourier self-deconvolution, or resolution enhancement, decreases the widths of infrared bands, thus allowing for separation and better identification of overlapping component bands (Surewicz and Mantsch, 1988).

To understand the concept of Fourier analysis, consider a typical Lorentzian infrared band whose lineshape has the analytical form:

$$\text{Equation A6 } A(\tilde{\nu}) = \frac{A_0 \gamma^2}{[\gamma^2 + (\tilde{\nu} - \tilde{\nu}_0)^2]}$$

$\tilde{\nu}$ = wavenumber
 $A(\tilde{\nu})$ = absorbance as a function of $\tilde{\nu}$
 A_0 = maximum absorbance
 $\tilde{\nu}_0$ = wavenumber at A_0
 γ = half-width at half-height

The band or lineshape can also be written as an infinite sum of sine and cosine terms.

The function $A(\tilde{\nu})$ in the interval $\tilde{\nu}_1 - \tilde{\nu}_2$ can be expressed by the Fourier series:

Equation A7 $A(\lambda) = \sum_{n=0}^{\infty} [a_n \sin(n\omega \lambda) + b_n \cos(n\omega \lambda)]$

where, $\omega = 2\pi/(\lambda_2 - \lambda_1)$

$$a_n = 2/(\lambda_2 - \lambda_1) \int_{\lambda_1}^{\lambda_2} A(\lambda) \sin(n\omega \lambda) d\lambda$$

$$b_0 = 1/(\lambda_2 - \lambda_1) \int_{\lambda_1}^{\lambda_2} A(\lambda) d\lambda$$

$$b(n > 0) = 2/(\lambda_2 - \lambda_1) \int_{\lambda_1}^{\lambda_2} A(\lambda) \cos(n\omega \lambda) d\lambda$$

Fourier analysis resolves the function $A(\lambda)$ into $\sin(n\omega \lambda)$ and $\cos(n\omega \lambda)$ functions, each with an appropriate weighting factor, to reveal its component wavelengths (Harris, 1987).

One way to increase separation between overlapping infrared bands is to obtain the n th-order derivative on the frequency domain of Fourier-transform of the absorbance spectrum. Typically, the power of the derivative is kept at the default value of 3 with a smoothing breakpoint in the range of 0 to 1 (Moffatt, Kauppinen, Cameron, Mantsch, and Jones, 1986). By varying the breakpoint, the derivative can be subjectively optimized when noting the noise level in the resulting Fourier-transformed band.

Fourier deconvolution involves manipulation of the cosine Fourier-transform of the absorption band (Surewicz and Mantsch, 1988):

Equation A8 $I(x) = F[A(x)] = 0.5A_0\gamma \cos(w\pi \lambda_0 x) \exp(-\pi\gamma x)$

where, x = distance of propagation

The Fourier-transform of the band is an exponentially decaying function of γ ; the wider

the absorbance band, the faster the decay of $I(x)$. Therefore, one would reduce the width of the band by decreasing the rate of decay of $I(x)$. This can be achieved by multiplying an exponentially increasing function $D(x) = \exp(2\pi\sigma x)$ with $I(x)$ to yield:

Equation A9
$$I'(x) = 0.5A_0\gamma \exp(2\pi\sigma x) \exp[-2\pi(\gamma-\sigma)x]$$

A new absorbance band $A'(x)$ is obtained by performing a reverse Fourier transform on $I'(x)$. The new halfwidth of $A'(x)$ is $\gamma' = \gamma - \sigma$. A resolution-enhancement factor can be defined as the ratio of γ/γ' (Surewicz and Mantsch, 1988). In contrast to Fourier-derivation, Fourier-deconvolution manipulates the absorbance domain of the Fourier series rather than the frequency domain.

While there are several advantages to Fourier-deconvolution and derivation, there are also some pitfalls. The major problems arise from the appearance of side lobes and the disproportionate enhancement of bands originating from water vapour or random noise.

A1.4 High-Pressure Fourier-Transform Infrared Spectroscopy

Over the past decade, it has become well recognized that the use of pressure as an added variable in vibrational spectroscopic studies is extremely powerful and practical (Wong, 1987). By increasing pressure on a sample, several spectral absorption features are affected such as frequency, intensity, bandshape and band splitting. By reducing the sample volume, pressure causes interactions, which are normally weak at ambient pressure, to increase in magnitude, leading to changes in the sample's absorption spectra. Consequently, details on structural and dynamical properties of the sample are revealed

that are not always obvious or available at ambient pressure.

In order to obtain infrared spectra at high pressure, a high-pressure optical cell and its optical link with the spectrophotometer are required. In the present study, an opposed diamond anvil cell is used to hold and compress the sample. Individual samples, which can be either in a liquid or solid state, are placed in a well of a stainless steel gasket between the parallel faces of two opposed diamond anvils. The cell becomes pressurized when the two opposed anvils are pushed towards one another.

Diamond is ideal for high pressure studies due to its hardness; it is able to withstand pressure as high as 100 kbar (Wong, Moffatt, and Baudais, 1985; Wong, 1987). For infrared and far infrared measurements, type IIa diamond is commonly used as it absorbs little in the infrared region, with the exception of the frequency range between 1930 and 2290 cm^{-1} , where it absorbs strongly.

The sample size and optical aperture in high-pressure infrared measurements are required to be small due to the small size of the high-pressure optical cells. The diameter of the cell's gasket well is approximately 0.3 mm. To maximize the signal-to-noise level of the spectra, a source beam condensing system is used. In this study, a NaCl lens system was used to condense the infrared beam onto the diamond anvil cell.

The hydrostatic pressure in the gasket of the diamond anvil cell is measured by monitoring the pressure shift of the infrared bands of crystalline quartz (Wong et al., 1985). The use of crystalline quartz as an internal pressure calibrant has several advantages; 1) Its infrared spectrum is relatively simple with sharp bands below the 1100 cm^{-1} frequency range; 2) Only a small amount is required to produce strong infrared

bands; 3) It is practically insoluble in water, making it ideal for high-pressure studies of aqueous systems; and 4) no structural phase transitions have been observed at room temperature in the pressure range up to 100 kbar (Wong et al., 1985).

Pressure calibration is performed by computer. By monitoring the change in frequency of the α -quartz phonon band, the pressure in the gasket can be calculated using the equation:

Equation A10
$$p = 0.01516(\Delta V)^2 + 1.2062(\Delta V)$$

where, p = pressure, kbar

Δv = change in wavenumber, cm^{-1}

v_o = wavenumber of α -quartz phonon
band at atmospheric pressure
= 694.1 cm^{-1}
(Wong, 1987)

A1.5 Application of High-Pressure Fourier-Transform Infrared Spectroscopy to Lipids

Lipids are relatively small molecules that consist of both a hydrophilic and hydrophobic moiety (Alberts, Bray, Lewis, Raff, Roberts, and Watson, 1989). They are, in general, water-insoluble, but highly soluble in organic solvents such as chloroform. Lipids play many biological roles; 1) they serve as fuel molecules, 2) energy stores, 3) signal molecules, and 4) components of membranes (Stryer, 1988). There are three major kinds of membrane lipids: phospholipids, glycolipids, and cholesterol.

Of interest in this study are glycolipids, which are sugar-containing lipids.

Glycolipids can be derived from either glycerol or sphingosine and are termed glyco glycerolipids and glycosphingolipids respectively. Glyco glycerolipids consist of a glycerol backbone, two fatty acid chains, and one or more sugar residues. The fatty acid chains generally consist of an even number of carbon atoms, typically between 14 and 24. Fatty acids may be saturated or unsaturated, with the configuration of any double bonds usually being cis (Stryer, 1988).

With glycosphingolipids, the amino group of the sphingosine backbone is acylated by a fatty acid and one or more sugar residues are linked to the primary hydroxyl group of the sphingosine backbone. The hydrophobic unit or the "tail" of glycolipids is the fatty acid chains with respect to glyco glycerolipids and the fatty acid chain and hydrocarbon chain of the sphingosine with respect to glycosphingolipids. The hydrophilic unit or "polar head group" of glycolipids consists of one or more sugar residues. The interfacial region of glyco glycerolipids is the C=O moiety that joins the hydrophobic region with the hydrophilic region. With respect to glycosphingolipids, the interfacial region is the amide group that is formed upon acylation of the sphingosine amine group with a fatty acid.

In an aqueous environment, the favoured structure for most glycolipids (and phospholipids) is a lipid bilayer. Given the amphipathic character of lipids, the structure of a bilayer is inherent in the structure of glycolipids (Alberts et al., 1989). Lipid bilayers are formed rapidly and spontaneously in water, with hydrophobic interactions being the major driving force. Moreover, between the hydrocarbon chains, there exists van der Waals attractive forces. Finally, electrostatic and hydrogen bonding attractions

exist between the polar head groups and water molecules.

Model biomembranes can be prepared by dispersing lipids in excess water to form closed multilamellar bilayers (Figure A1.6). By using high-pressure infrared spectroscopy, several structural and dynamic properties of model biomembranes can be revealed. By reducing the volume, weak interactions such as van der Waals and electrostatic forces are greatly enhanced and reflected in the resulting infrared spectra (Wong, 1987).

Figure A1.6 - Schematic representation of a multilamellar vesicle (from Warren, 1987, p25).

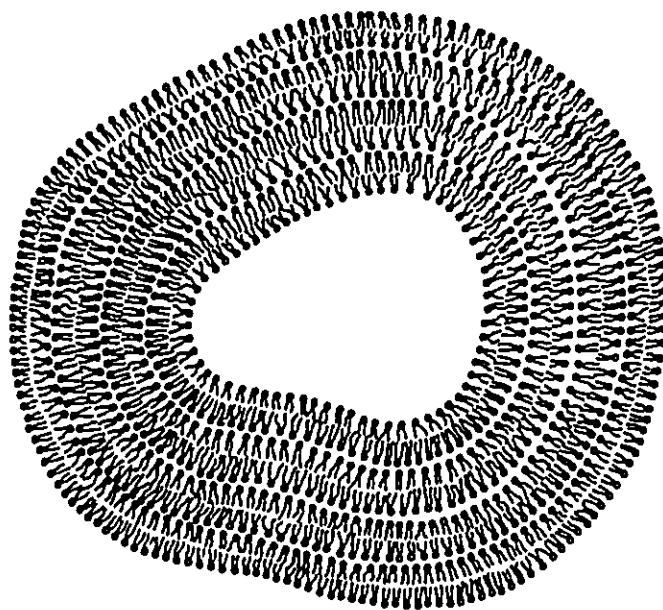


Figure A1.6

Under ambient pressure conditions, the stretching frequencies of hydrogen bond forming groups, such as O-H and N-H decrease in frequency with respect to those of non-hydrogen bonded O-H and N-H groups. This is due to the fact that hydrogen bonding causes an elongation of the O-H and N-H bonds, thus weakening these bonds and causing their absorption frequencies (and bond energy) to lower in magnitude. When pressure is applied to hydrogen bonded groups, the hydrogen bonding is enhanced and their stretching frequencies decrease further in magnitude (Wong, 1987). The stronger the hydrogen bonding, the greater the pressure-induced frequency shift.

High-pressure infrared spectroscopy is extremely powerful in revealing structural and dynamic details on lipid hydrocarbon chains. The conformational and orientational order of the hydrocarbon chains ultimately determines the "fluidity" of the lipid and bilayer. There are several variables that can affect conformational and orientational order of hydrocarbon chains such as, 1) chain length, 2) chain unsaturation/saturation, 3) interdigitation of the chains, 4) hydrogen bonding and electrostatic forces between the head groups and water, 5) temperature, 6) the presence of exogenous molecules, and 7) pressure.

By applying external pressure to a lipid multilayer, the normally weak van der Waals forces that exist between the hydrocarbon chains are greatly enhanced (Wong, 1987). By laterally compressing the hydrocarbon chains perpendicularly to the chain axis, the conformational disorder, the reorientational fluctuations and the twisting/torsion motions of the chains can be ordered and dampened. Spectroscopically, this affects the vibrational modes that are due to the hydrocarbon chains (Wong, 1984, 1987, in press;

Wong and Mantsch, 1985, 1988). If the equilibrium orientations of neighbouring chains are nonequivalent (i.e., perpendicular), the dampening of the chain fluctuations and motions induces a correlation field splitting of the vibrational modes of the hydrocarbon chains (Wong, 1984, 1987, in press; Wong and Mantsch, 1985, 1988). If the chains are more orientationally disordered, a higher pressure is required to stop these fluctuations and motions. Consequently, the pressure at which the correlation field splitting appears is higher. The magnitude of the correlation field splitting pressure can, therefore, be used to determine the order/disorder dynamics of the hydrocarbon chains in lipid bilayers.

The explanation of correlation field splitting is based on the first order perturbation theory, which states that the exciton level of a lipid bilayer system with two nonequivalent (i.e., perpendicular) hydrocarbon chains per unit cell (a and b chains) will split into two branches (Wong, in press). Consequently, the vibrational mode will split into two with the frequencies being:

$$\text{Equation A11 } \omega_1 = \omega_0 + \frac{1}{8\pi^2 \omega_0 c} \left(\sum_{i=1}^{2N} \left(\frac{\partial^2 U_0}{\partial q_i^2} \right) + \sum_{j=1}^N \left(\frac{\partial^2 U_1}{\partial q_a \partial q_{aj}} \right)_0 + \sum_{j=1}^N \left(\frac{\partial^2 U_2}{\partial q_a \partial q_{bj}} \right)_0 \right)$$

$$\text{Equation A12 } \omega_2 = \omega_0 + \frac{1}{8\pi^2 \omega_0 c} \left(\sum_{i=1}^{2N} \left(\frac{\partial^2 U_0}{\partial q_i^2} \right) + \sum_{j=1}^N \left(\frac{\partial^2 U_1}{\partial q_a \partial q_{aj}} \right)_0 - \sum_{j=1}^N \left(\frac{\partial^2 U_2}{\partial q_a \partial q_{bj}} \right)_0 \right)$$

Where, ν_0 = wavenumber of each chain vibrational mode

U_0 = pair perturbation potential function

U_1, U_2 = pair perturbation potential function between equivalent and nonequivalent, respectively

q = normal coordinate

a, b = site a, b of the hydrocarbon chain

The first term of the equation is the potential energy changes of the vibrational modes of the individual chains arising from the perturbation of neighbouring stationary chains. The second and third terms are the potential changes from the perturbation of intramolecular displacements of the orientationally equivalent and nonequivalent chains, respectively. The correlation field splitting, which is the splitting of each frequency of each chain into ν_1 and ν_{II} components is:

Equation A13

$$\Delta\nu = \frac{1}{4\pi^2 \nu_0 c} \sum_{j=1}^N \left(\frac{\partial^2 U_2}{\partial q_a \partial q_b} \right)_0$$

If the hydrocarbon chains in a bilayer system are orientationally parallel, the third term in equations A11 and A12 and $\Delta\nu$ in equation A13 would be equal to zero. Thus, there would be no correlation field splitting in the hydrocarbon chain vibrational modes. There would also be no correlation field splitting in the spectra under two other circumstances: 1) The conformation of the hydrocarbon chains is so highly disordered that the coupling of the vibrational modes between neighbouring chains is random and weak; or 2) The conformation of the hydrocarbon chains is highly ordered but the orientations of the chains are disordered due to reorientational fluctuations and twisting and torsion motions. In such a case, rather than a correlation field splitting in the vibrational bands, a broadening of the bands is observed (Wong, in press).

High-pressure Fourier-transform infrared spectroscopy provides an excellent method to study lipid structure and dynamics by analysis of the pressure dependencies of the band frequencies, widths, intensities, shapes, and splittings.

Figure A2.1 - Schematic representation of methanolysis/acetylation reaction of SGG to obtain fatty acid methyl esters (FAMES) and monoalkyl glycerol diacetates (MAGDs).

APPENDIX TWO

METHANOLYSIS/ACETYLATION REACTIONS OF SGG

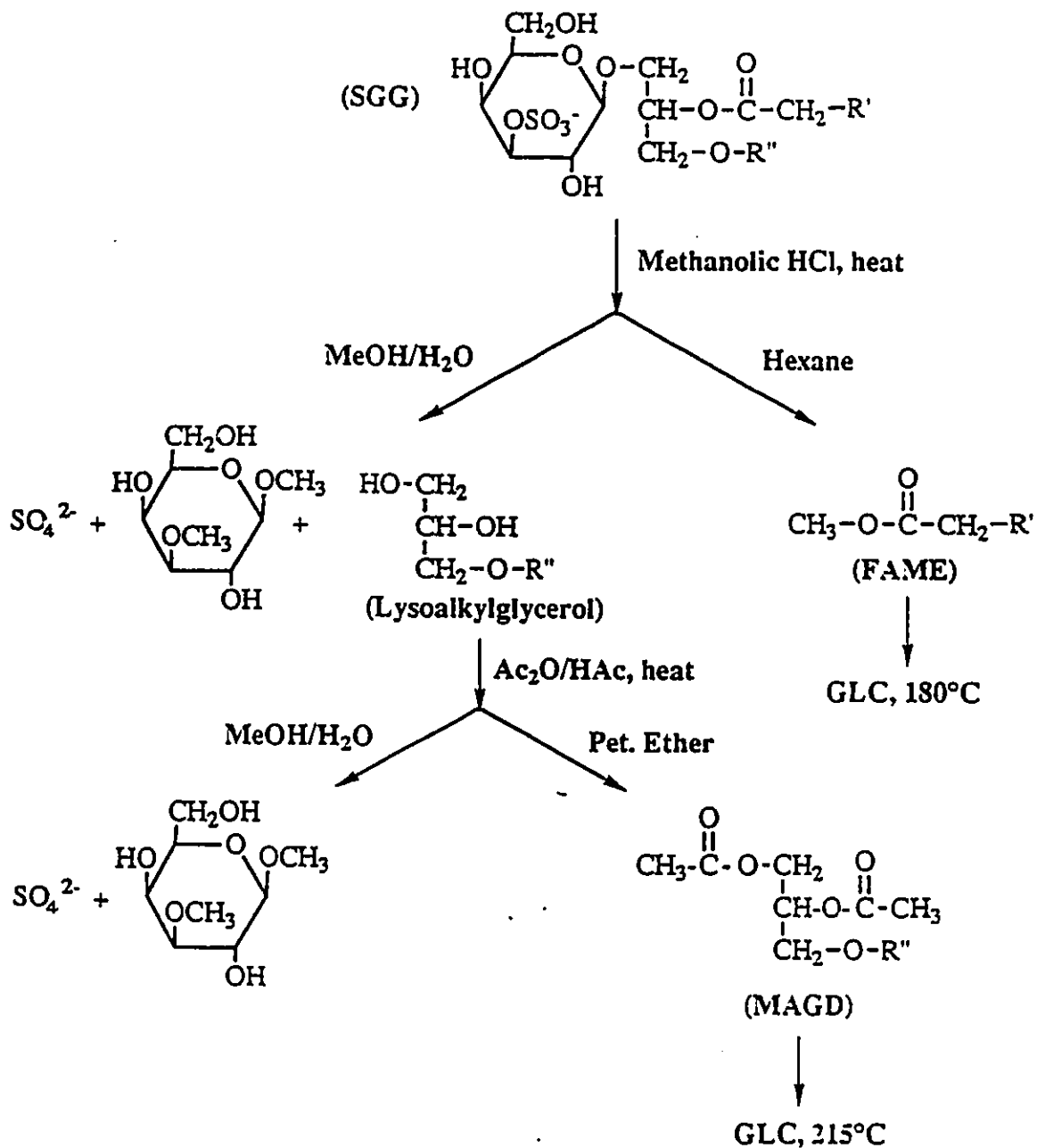


Figure A2.1

APPENDIX THREE

THEORY OF GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography involves partitioning components of a mixture in the vapour state between a mobile stable gas phase and a stationary non-volatile liquid phase coated on an inert support (Harris, 1987). A volatile liquid sample is injected into a hot injector port, which vapourizes the sample. The injector port temperature is maintained at 50°C higher than the column temperature (in this study, 180°C and 215°C) to promote vapourization of the sample. An inert carrier gas (N₂) serves as the mobile phase and sweeps the sample into the column. The components pass through the column while being partitioned between the mobile phase and stationary phase. The components flow at different rates according to their properties, resulting in separation of each component. The retention time of each component depends on several factors: 1) polarity of component, 2) polarity of stationary phase, 3) polarity of mobile phase, 4) column temperature, 5) flow rate of mobile phase, and 6) capacity of stationary phase. Once the components have passed through the column containing the stationary phase, the gas stream flows through a flame ionization detector, which outputs a signal to a chart recorder as the components emerge from the column. The detector is also kept at a higher temperature than the column to promote solute vapourization (Harris, 1987).

Gas chromatography columns can be made of stainless steel, aluminum, or glass as in the case of this study. Most GLC columns contain a nonvolatile liquid dispersed on fine particles of a solid "inert" support. Ideally, the support should be comprised of small, strong, uniformly shaped particles with a surface area that is proportionally large.

A uniform particle size results in an increase in resolving power for the column, while a small particle size reduces the time required for solute equilibration, thus increasing column efficiency (Harris, 1987). However, if the particle size is too small, the column resistance causes the flow rate to be reduced to a useless value.

The stationary liquid phase used in GLC is determined by the type of solutes being separated. The rule "like dissolves like" is kept in mind when selecting a stationary phase. There is generally a wide selection of stationary phases (i.e., SP-2330 = cyanopropyl silicone) that may be suitable for the solute separation, so long as the class of solute is effectively retained by the stationary phase such that resolution is good.

Inside the flame ionization detector (Figure A3.1), the column eluate is combined with H_2 and air and burned in a flame. $CH^\bullet$ radicals can be produced from the carbon atoms of organic compounds, which in the hydrogen-oxygen flame go on to produce CHO^+ ions (Harris, 1987). Although only one in 10^5 carbon atoms produces an ion, the CHO^+ production is even and strictly commensurate to the number of susceptible carbon atoms entering the flame (Harris, 1987). The CHO^+ ions produced in the flame carry a current to the cathodic collector, which outputs the detector response onto a chart recorder.

Figure A3.1 - Schematic diagram of a flame ionization detector for a gas-liquid chromatograph (from Harris, 1987, p640).

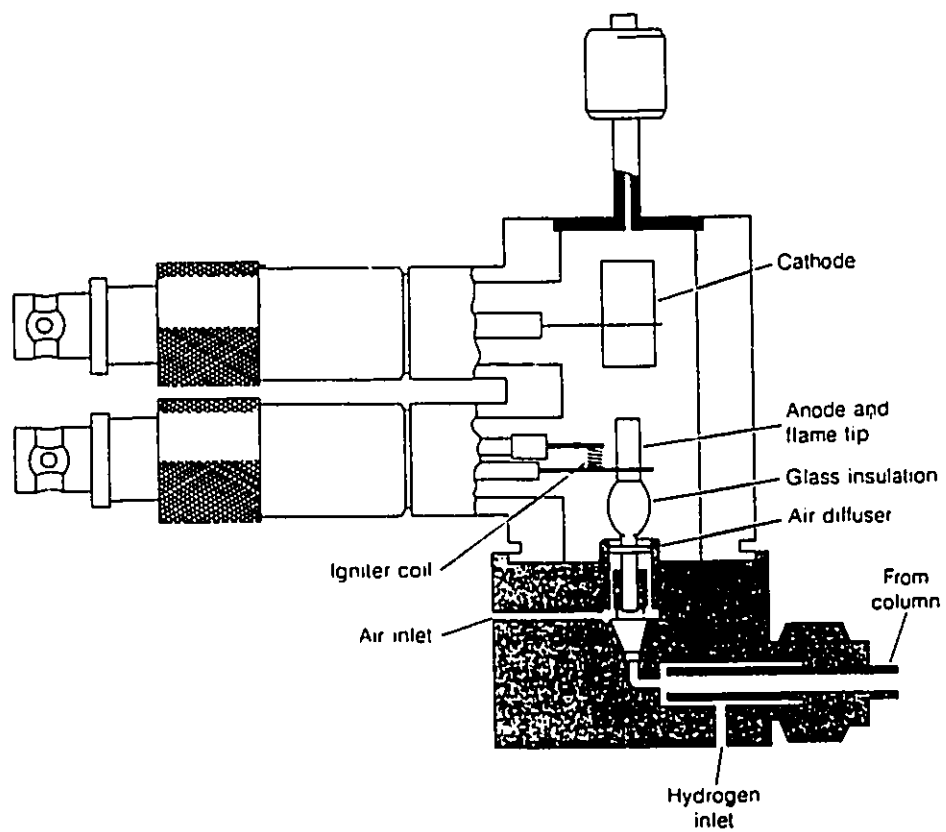


Figure A3.1

The components from the mixture are identified by their retention times on the chromatogram. Standards are often run along side a mixture or under the same conditions to assist in the identification. Ultimately, however, the chemical and physical properties of the isolated material have to be determined for final identification, although this is generally not feasible for every analytical GLC run. In practice, the general chemical class of the analyzed component is normally known from the procedure used in its preparation or from TLC and spectral analysis.

The retention times of the GLC peaks can be used to determine hydrocarbon chain types, chain lengths, number of double bonds, and the kind of functional groups present. Under conditions of constant temperature and flow rate, the retention time (t_n) for a component with n carbons is directly related to the retention times (t_{n+1} and t_{n-1} , respectively) of the component with $n+1$ and $n-1$ carbon atoms:

Equation A3.1
$$t_{n+1}/t_n = t_n/t_{n-1} = F \quad F = \text{CH}_2 \text{ separation factor}$$
$$= \text{constant}$$

The separation factor for 2CH_2 groups is:

Equation A3.2
$$t_{n+2}/t_n = t_n/t_{n-2} = F^2$$

Generally, for $x\text{CH}_2$ groups, the relationship is:

Equation A3.3
$$t_{n+x}/t_n = F^x \quad x = 1, 2, 3, \dots$$

Similarly, the number of double bonds in the hydrocarbon chain of an unknown can also be determined by comparing the relative retention times of standards with known number of double bonds in their hydrocarbon chains and establishing the $\text{C}=\text{C}$ separation factor (R). The retention time (t_d) for a component with d double bonds is directly

related to the retention times (t_{d+1} and t_{d-1} , respectively) of the components with $d+1$ and $d-1$ double bonds, provided that they have the same number of carbon atoms:

Equation A3.4 $t_{d+1}/t_d = t_d/t_{d-1} = R$ $R = \text{C=C separation factor}$
 $= \text{constant}$

The separation factor for 2 C=C bonds is:

Equation A3.5 $t_{d+2}/t_d = t_d/t_{d-2} = R^2$

Generally, for $x\text{C}=\text{C}$ bonds, the relationship is:

Equation A3.6 $t_{d+x}/t_d = R^x$ $x = 0, 1, 2, \dots$

The separation factors F and R are dependent on only the temperature of the column and the type of stationary phase, but is independent of chain length or the type of compound (Kates, 1986). It is therefore practical to work, not with retention time (t_n , t_d), but with relative retention time with respect to a suitable standard. Relative retention times are a function of the column temperature and stationary phase.

Quantitation of each fraction is possible by measuring each peak area. Each peak is basically a Gaussian distribution curve with an area determined by the equation:

Equation A3.7 $A = \sqrt{2\pi}\sigma h = 2.507\sigma h$ $A = \text{area of peak}$
 $h = \text{height of peak}$
 $\sigma = \text{standard deviation}$

However, the standard deviation, σ , is proportionate to the peak width, w . Therefore,

Equation A3.8 $\sigma = kw$ $k = \text{constant}$

By substituting equation A3.8 into equation A3.7, we get:

Equation A3.9 $A = kwh$

The peak area is therefore, proportional to the peak height and width.

The method used in this study to determine peak area is the peak height x retention time method (Kates, 1986). This method is based on the fact that the standard deviation, σ , which is directly proportional to the peak width, w , is a linear function of the retention time, t_n . By substituting for w in equation A3.9, we get:

Equation A3.10 $A = k' \cdot h \cdot t_n$ $k' = \text{constant}$

This method can be used to determine peak area percentages with k' being cancelled out in the equation:

Equation A3.11
$$\%A = \frac{k' \cdot h_n \cdot t_n}{\sum k' \cdot h_n \cdot t_n} \times 100 = \frac{h_n \cdot t_n}{h_1 t_1 + h_2 t_2 + h_3 t_3 \dots} \times 100$$

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