MIXING OF SPHINGOLIPIDS WITH PHOSPHOLIPIDS IN LIPID BILAYER MEMBRANES: CALORIMETRIC AND FLUORESCENCE STUDIES

© Michael A. Gardam

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Master of Science.

Department of Biochemistry

McGill University

Montréal, Québec

Canada

Mixing of Sphingolipids with Phospholipids in Lipid Bilayer Membranes: ~~Calorimetric and Fluorescence Studies~~

Abstract:

It has been previously theorized that lipids possessing different headgroup structures mix non-ideally in liquid crystalline bilayers, due to either the attractive or repulsive interactions of their headgroup moieties. I have measured the relative energies of interactions between lipids with different headgroups, using a new technique that measures the equilibrium distribution of exchangeable fluorescent lipids between different bilayer environments. The results obtained from these studies suggest that lipids with different headgroup structures may mix more nearly ideally in liquid-crystalline bilayers than previously suggested on the basis of more traditional, less direct techniques.

Phospho- and sphingolipids bearing two hydrocarbon chains of considerably different length, termed 'asymmetric species', have recently been shown to exhibit solid-solid and, in one case, liquid crystalline-liquid crystalline (Mason, 1988) phase separations in binary mixtures with phospholipids that carry two identical acyl chains. I have studied binary mixtures of 'asymmetric' phospho-and sphingolipid species with the 'symmetric' species dipalmitoyl phosphatidyl choline (DPPC) and have found such mixtures to be miscible, albeit non-ideally, in the liquid-crystalline phase. The long-chain sphingolipids were found to be very similar to shorter-chain sphingolipids in their miscibilities with DPPC, in both the gel and the liquid-crystalline state, suggesting that 'asymmetric' sphingolipids will exhibit no special tendency to laterally segregate from 'symmetric' species in liquid-crystalline membranes.

Résumé:

Il a été proposé que des lipides, possedant des têtes polaires de structures différentes, forment un mélange non-idéal en bicouche à l'état fluide due aux intéractions de répulsion ou d'attraction entre les groupes de leur tête polaire. J'ai mesuré les énergies relatives d'intéractions entre des lipides ayant différentes têtes polaires, en utilisant une nouvelle technique qui compare la distribution à l'équilibre de lipides échangeables fluorescents entre differents environnements de bicouches lipidiques. Les résultats obtenus grace à ces études suggèrent que des lipides, ayant differentes tetes polaires, pourraient se mélanger de façon plus idéale, à l'intérieur de bicouches à l'état fluide, que le laissaient entendre des observations basées sur des méthodes plus traditionnelles et moins directes

Il a été observé, récemment, que les phospho- et sphingolipides portant deux chaines d'hydrocarbones de longueur considérablement différentes, nommées 'espèces asymetriques', presentent des séparations de phase solide-solide et, dans un cas particulier, fluide-fluide (Mason, 1988) en mélanges binaires de phospholipides portant deux chaînes acyles identiques. J'ai etudié des mélanges binaires de phospho- et le sphingolipides 'asymetriques' avec l'espèce 'symétrique' dipalmitoyl phosphatidylcholine (DPPC), et j'ai observé que ce type de mélange était miscible, bien que de façon non idéale, à l'état fluide. Les sphingolipides à longue chaine et les sphingolipides à courte chaîne présentent de grandes similitudes quant à leur miscibilité avec le DPPC, à l'état gel comme à l'état fluide, suggerant que les sphingolipides 'asymétriques' ne montrent pas de tendance particuliere à se séparer des espèces 'symetriques' en ce qui concerne les membranes fluides.

TABLE OF CONTENTS

CHAPTER TWO: Partitioning of Exchangeable Fluorescent Phospholipids and Sphingolipids between Different Lipid Bilayer Environments

ACKNOWLEDGEMENTS:

Foremost, I would like to thank my supervisor, Dr. J.R. Silvius for his guidance, support, eclectic taste in music, and sense of humour throughout my graduate studies.

I am grateful for the advice and friendship of the members of the Department of Biochemistry and elsewhere, in particular: Carol Miyamoto, Rania Leventis, Pamela Brown, Scott Munzer, Serge Shahinian, Elana Swartzman, Carlo Polvani, Joey Ilovitch, Dr. R.F MacKenzie, and Dr. Wendy Findlay.

I would particularly like to thank: my parents for their support, financial and otherwise, Kevin MacKenzie for his friendship, and Valérie Sales for her friendship and for the translation of the abstract into French.

This work was financially supported through a Medical Research Council of Canada Studenship.

PUBLICATIONS:

Papers:

Gardam, M.A., Itovitch, J.J. and Silvius, J.R. (1989) Biochemistry 29, 884-893.

Gardam, M.A. and Silvius, J.R. (1989) Biochim. Biophys. Acta 980, 319-325.

Abstracts:

Gardam, M.A., Itovitch, J.J., and Silvius, J.R. (1988) Biophys. J. 53, 503a

Leventis, R., Gardam, M.A., and Silvius, J.R. (1987) Biophys. J. 51, 43a.

LIST OF TABLES AND FIGURES:

CHAPTER ONE:

CHAPTER TWO:

CHAPTER THREE:

LIST OF ABBREVIATIONS:

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
PA	phosphatidic acid
PI	phosphatidylinositol
DPPC, DPPE etc...	1,2-dipalmitoyl-sn-glycero-3-phosphocholine, phosphoethanolamine etc ...
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DEPC	1,2-dielaidoyl-sn-glycero-3-phosphocholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phosphoserine
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
DSC	differential scanning calorimetry
FT-IR	Fourier Transform Infra-red Spectroscopy
NMR	Nuclear Magnetic Spectroscopy
ESR	Electron Spin Resonance Spectroscopy
C(18):C(0) PC	1-stearoyl-2-lyso-sn-glycero-3-phosphocholine

C(18):C(2) PC	1-stearoyl-2-acetyl-sn-glycero-3-phosphocholine
C(18):C(10) PC	1-stearoyl-2-caproyl-sn-glycero-3-phosphocholine
C(18):C(12) PC	1-stearoyl-2-lauroyl-sn-glycero-3-phosphocholine
C(18):C(14) PC	1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine
C(18):C(16) PC	1-stearoyl-2-palmitoyl-sn-glycero-3-phosphocholine
(8-bimane)-	8-(bimanylmercapto)octanoyl-
(8-bimane)-PC	1-palmitoyl-2-(8-(bimanylmercapto) octanoyl)-PC
(12-DABS)-18 PC	1-hexadecanoyl-2-(12-[[4-[[4-(dimethylamino) phenyl]azo]phenyl]sulfonyl]methylamino octadecanoyl)-sn-glycerol-3-phosphocholine
(11-DMCA)-	11-((7-dimethylaminocoumarin-3-yl)carbonyl) methylaminoundecanoyl-
(11-DMCA)-PC	1-palmitoyl-2-(11-((7-dimethylaminocoumarin-3-yl)carbonyl)methylaminoundecanoyl)-PC
DPHP-	3-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)propanoyl-
EDTA	ethylenediaminetetraacetic acid trisodium salt

Egg PC	phosphatidylcholine from egg yolk
Egg PE (Egg PG)	phosphatidylethanolamine (phosphatidyl glycerol) prepared by transphosphatidylation of egg yolk PC
LUV	large unilamellar vesicle(s)
O-methyl PA	1,2-dioleyl-sn-glycero-3-phosphomethanol
Tes	tris(hydroxymethyl)methanesulfonic acid sodium salt
TNBS	trinitrobenzenesulfonic acid sodium salt
N-24:0-cerebroside	N-lignoceroyl galactocerebroside
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
12:0/24:0-PC	1-Lauroyl-2-lignoceroyl phosphatidylcholine
N-24:0-sulfatide	N-lignoceroyl 3'-sulfo galactocerebroside.

CHAPTER ONE: Introduction

1.1 Preface:

The fluid-mosaic model of biological membranes (Singer and Nicholson, 1972) has emerged as the accepted model of membrane structure. In interpreting this model, lipids are often assigned a purely structural role, maintaining the barrier properties of the membrane and serving as a fluid, yet ordered matrix into which the membrane proteins are inserted. In this view, the important functional properties of the membrane are attributed exclusively to the protein component.

In fact, however, this view of membrane structure and function may be seriously oversimplified in some important respects. Recent research has indicated that the functional roles of the lipid component in biological membranes are considerably more complex than previously envisioned. Almost all biological membranes contain a wide array of lipids, the relative proportions of which are tightly regulated. A clear relationship between membrane lipid composition and membrane function has been established in a variety of studies that have demonstrated a significant role of lipid structure in modulating lipid-protein and protein-protein interactions in artificial and biological membranes (Sandermann, 1978, Zwaal, 1978). Some major classes of membrane lipids have also been shown to form non-bilayer structures that may be present during biologically important processes such as membrane fusion (Cullis and De Kruijff, 1979). Other membrane lipids have been shown to be involved in pathways for information transfer within or across membranes. Phosphatidyl inositol (PI) and its mono- and diphosphorylated derivatives, for example, have been shown to be involved in information propagation from mammalian cell surfaces to the cytoplasm. Upon stimulation of specific cell surface receptors, phosphorylated derivatives of PI are rapidly converted into a series of potent second messengers (including inositol triphosphate and diacylglycerol) which induce dramatic and widespread cellular responses such as calcium mobilization and kinase activation (Berridge and Irvine, 1984; Cockcroft and Gomperts, 1985). In the light of these facts, it seems necessary to broaden

our picture of the role of membrane lipids from that of a purely structural component to one in which they are given a more active role in membrane function.

The aim of my thesis is to contribute to the existing body of knowledge concerning how lipid structure can affect the gross organization of lipid mixtures, and how this organization may be related to the physiological function of membranes. As discussed later in this chapter, my ultimate goal is to evaluate how well (or how poorly) the lipid phase in a biological membrane can be modeled as a homogeneous two-dimensional 'solution' of different lipid species, whose distinctive properties contribute to the overall properties of the membrane only by 'averaging' with those of the other lipid species present. The alternative view, that certain types of lipids can form laterally segregated 'clusters' or even domains in the membrane plane, has been put forward and deserves serious evaluation, if only to dismiss the likelihood of such behaviour, in view of the potential influence of such behaviour on membrane function.

I will therefore present studies examining the effects of two aspects of lipid structure, namely polar headgroup structure and acyl chain length 'mismatch', on lipid-lipid interactions in experimental systems. The associations between lipid headgroups will be studied using a new approach involving the equilibration of fluorescent-labelled lipids between lipid vesicles of different compositions. The effect of acyl chain length 'mismatch' on lipid mixing will be studied in binary systems combining phosphatidylcholine with various phospho- and sphingolipids bearing highly asymmetric hydrocarbon chains (i.e. two hydrocarbon chains of considerably different length). Such lipid species have recently been suggested to form discrete domains within biological membranes and hence, may have a significant role to play in membrane function.

1.2 Lipid Structures:

Animal cell membranes contain a wide variety of lipid species, almost all of which however can be grouped into three classes; the glycerol-based phospholipids, the sphingolipids, and the sterols.

Biologically occurring phospholipids have the following basic structure: a glycerol backbone substituted with long fatty acyl chains at positions C-1 and C-2, linked through a phosphodiester linkage at C-3 to a polar alcohol. The most common polar lipid headgroup structures are shown in Figure 1. Within a given headgroup class, e.g., the phosphatidylcholines, there is a large degree of variation with respect to fatty acyl chain length and degree of unsaturation. The lipids of human erythrocyte membranes, for example, are composed of fatty acyl chains which vary in length from 16 to 24 carbons and in degree of saturation from 0 to 6 double bonds (van Deenen and de Gier, 1974). Long-chain saturated fatty acyl chains are found predominantly at the C-1 position of the glycerol backbone and unsaturated fatty acyl chains at the C-2 position. Phospholipids are by far the most widely distributed of the membrane lipids and comprise the major lipid component of animal cell membranes (see Table 1).

The sphingolipids are a highly heterogeneous class of lipids based primarily on the long-chain bases sphingosine, dihydrosphingosine, and 4-hydroxysphingosine. In all sphingolipids, the C-2 amino group of the sphingosine base is linked to a fatty acyl chain creating an amphipathic molecule with two long hydrophobic chains, an arrangement similar in overall structure to the diacyl glycerol moiety of phospholipids. The N-linked acyl chains, typically 16 to 24 carbons in length, are either saturated or mono-unsaturated, and may be hydroxylated at C-2. Three acyl chains, 16:0[1], 24:0, and

---

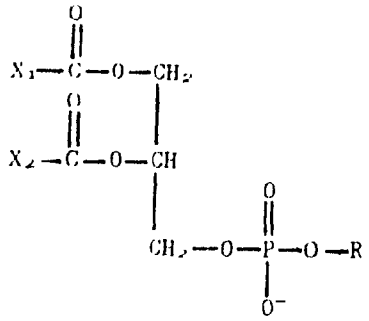
[1]The acyl chain length and position, confirmation, and number of unsaturated bonds are often designated by a shorthand notation. For example, an 18 carbon chain with a cis double bond between C_9 and C_{10} would be written 18:1 (Δ^9). The Δ notation is often not included unless the double bond is of specific interest.

Figure 1: Lipid Structures (at physiological pH)

Phospholipids:

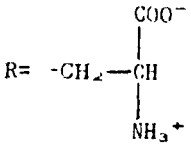
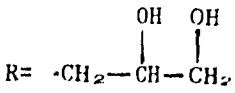
Backbone:

Diacylglycerol phosphate



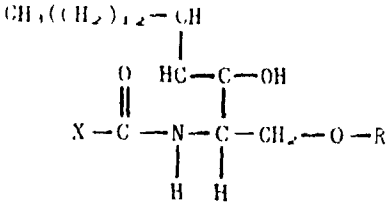
X_1 - saturated acyl chain
X_2 - unsaturated acyl chain

Headgroup:

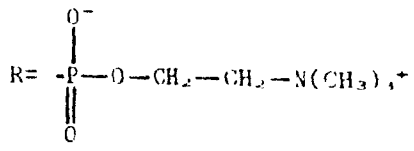
	Lipid:
R= -H	PA
R= 	PS
R= $-CH_2-CH_2-NH_3^+$	PE
R= $-CH_2-CH_2-N(CH_3)_3^+$	PC
R= 	PG

Sphingolipids:

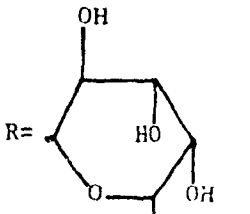
Sphingosine:



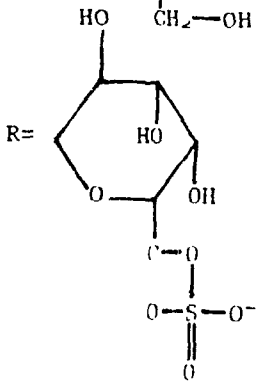
Y - acyl chain



Sphingomyelin



Galactosyl ceramide (a cerebioside)



Galactosyl ceramide sulfate (a sulfatide)

Table 1.

The Lipid Composition (in weight %) of Various Biological Membranes (from Vance and Vance (1985))

Lipid	Erythrocyte*	Myelin*	Mitochondria**	Endoplasmic Reticulum**
Cholesterol	23	22	3	6
Phosphatidyl Ethanolamine	18	15	35	17
Phosphatidyl Choline	17	10	39	40
Phosphatidyl Serine	7	9	2	5
Sphingomyelin	18	8	--	5
Glycosphingo-lipids	3	28	--	27

* from human sources
** from rat liver

24:1, account for the majority of sphingolipid N-acyl chain groups in human erythrocyte membranes (van Deenen and de Gier, 1974). Similarly, three acyl chain species (18:0, 24:0, and 24:1) account for >50% of the hydrocarbon chains found in myelin (Abe and Norton, 1979). Some representative sphingolipids are shown in Figure 1. (For the purpose of clarity in this thesis, I will use the term 'phospholipids' only to describe species based on diacyl glycerolphosphate and will include sphingomyelin with the sphingolipids). Although less prevalent in animal cell membranes than are phospholipids, sphingolipids can be found in relatively high concentrations in cell plasma membranes, particularly in specific tissues such as myelin (Table 1).

Cholesterol is the major sterol component of mammalian cellular membranes. In addition to the 4-membered steroid ring system, cholesterol possesses a 3-ß-hydroxyl group and a branched hydrocarbon chain attached at opposite ends of the steroid nucleus. Cholesterol is generally found in cell plasma membranes and is less prevalent in intracellular membranes, although endosomes (which are derived from the plasma membrane) are a notable exception (Heiniger et al., 1976; Evans and Hardison, 1985; Steck et al., 1988). The cholesterol content of plasma membranes varies considerably from tissue to tissue; in some membranes, such as those of human erythrocytes and particularly myelin, it is a major component, approaching equimolar ratios with phospholipids (van Deenen and de Gier, 1974).

To study the behaviour of a particular lipid species in a system as heterogeneous as a biological membrane would be an arduous, if not impossible task. Therefore, one is often obliged to study simpler model systems, typically bilayers with relatively few lipid and/or protein components, with the eventual goal of extrapolating the data to describe the biological system. While this goal has yet to be fully realized in many cases, an enormous amount of data has been amassed concerning the nature of lipid-lipid interactions and how these interactions may affect membrane structure and function.

One of the more intriguing theoretical implications of nonideal interactions between different lipid species is the possible forma-

tion of separate domains composed of discrete lipid species in multicomponent liquid-crystalline bilayers. Such domains have been suggested to participate in several important biological functions including the binding of ligands to the membrane surface (Cuatrecasas, 1973; Haywood, 1974; Revesz and Greaves, 1975; Mullin et al., 1976; Holmgren et al., 1980; Critchley et al., 1981), the formation of membranes with unusual morphology (Curatolo, 1987), the enhancement of membrane stability (Schmidt et al., 1978) and the maintenance of bilayer continuity at lipid-protein interfaces (Gerritsen et al., 1979).

I will now discuss the physical behaviour of the common phospho- and sphingolipids in aqueous solution, both as pure compounds and in simple mixtures with one another.

1.3 The Effect of Lipid Structure on Lipid-Lipid Interactions:

1.3.1 Introduction of Lipid Phase Behaviour:

Before discussing specific lipid-lipid interactions it is necessary to introduce the concept of lipid phase behaviour. Membrane lipids can exist in several *temperature-dependent* states. At low temperatures, some fully hydrated phospholipids can form one or more gel or crystalline phases (denoted as L_{β}, L_{β}' or L_c). In these phases, the acyl chains are tightly packed with extensive van der Waals' contacts and relatively little chain motion. At higher temperatures, gel-state bilayers typically convert to a fluid state called a liquid-crystalline phase (denoted as L_{α}) in which the acyl chains are no longer tightly packed and exhibit increased molecular motion. This particular phase transition is often colloquially referred to as 'melting'. The temperature at which a given hydrated lipid undergoes this transition (denoted as T_M) is dependent on the stability of the system in the gel phase: *the more stable the gel* phase, the higher the T_M. T_M tends to increase with increasing acyl chain length, as this results in additional van der Waals' contacts between adjacent lipids, which are stronger in the gel state.

Conversely, unsaturated or branched acyl chains disrupt acyl chain packing in the interior of the bilayer and hence lower T_M. For single pure lipid species, the gel-to-liquid-crystalline phase transition is strongly cooperative and behaves as a classical first-order phase transition (Albon and Sturtevant, 1978). By contrast, a heterogeneous mixture of phospho- and sphingolipids, such as those found in biological membranes, will characteristically have a broad gel-to-liquid crystalline phase transition.

For some lipids, raising the temperature still further may result in another phase transition, from the liquid-crystalline phase to a non-bilayer (e.g., hexagonal) phase. Such transitions have been observed for unsaturated phosphatidylethanolamines, cardiolipins, and phosphatidic acids under physiological or near-physiological conditions (Cullis and de Kruijff, 1978). The tendencies of different lipid species to form nonlamellar *vs.* lamellar phases is often explained in terms of their different 'dynamic shapes': the type of aggregate formed by a specific lipid species in aqueous solution is dependent on the ratio between the volumes and cross-sectional areas of the headgroup and the acyl chains (Israelachvili *et al.*, 1976). These latter properties in turn, depend on the length and degree of unsaturation of a lipid's acyl chains and the balance of repulsive and attractive forces (electrostatic, steric, dipolar and hydrogen-bonding) between the headgroups of adjacent lipids.

1.3.2 The Effect of Headgroup Structure on Lipid-Lipid Interactions

1.3.2.1 Pure Lipid Systems

Lipids with identical acyl chains but different headgroups often exhibit remarkably different phase behaviour. For example, the dipalmitoyl phospholipids listed in Table 2 have considerably different gel to liquid crystalline transition temperatures. Several factors have been suggested to influence the transition temperatures of phospholipids with different headgroups, including headgroup size

Table 2.

The Main Transition Temperatures (T_M) of Several Dipalmitoyl Phospholipids

Lipid	Charge*	≈ T_M (°C)	Reference
DPPA	-1	65	Blume and Eibl (1981)
DPPE	0	63	ibid.
DPPS	-1	54	Gove et al. (1981) MacDonald et al. (1976)
DPPC	0	41	Van Dijck et al. (1976) Phillips et al. 1970)

*charge at neutral pH

and charge at physiological pH (Bach et al., 1978; Van Dijck et al., 1978; Browning, 1981a,b; for a contrasting report, see Eibl, 1983), and ability to donate and accept hydrogen bonds (for recent reviews, see Boggs, 1980, 1987).

Hydrogen-bonding interactions have been suggested to promote tighter associations between the headgroups of species such as PE, PS and PA, which may account for the higher transition temperatures of these lipids (Boggs, 1980, 1987). Several groups have presented data concerning the effect of various physical parameters on phosphatidylethanolamine phase transitions which support this concept (Papahadjopoulos and Weiss, 1969; Seddon et al., 1983). Similar experiments have been performed on pure PS species (MacDonald et al., 1976; van Dijck et al., 1978; Cevc et al. 1981). Cevc et al. (1981) showed that PS exhibits its maximum gel-liquid crystalline phase transition temperature at low pH, where it exists as a neutral molecule. At neutral pH, a lower transition temperature is measured at low salt concentrations; however, at high ionic strength, where the negative charge of PS is largely shielded, the transition temperature increases to a value similar to that of the neutral (low pH) species and to that of the corresponding PE (Cevc et al., 1981). These results suggest that the transition temperature of PS is significantly influenced both by electrostatic factors and by hydrogen-bonding interactions between headgroups.

Pure PA systems have also been studied in effort to explain the high phase transition temperature of this lipid at physiological pH (Jacobson and Papahadjopoulos, 1975; Blume and Eibl, 1979; Eibl and Blume, 1979). Boggs (1987) suggests that the monoprotonated form of PA can participate in intermolecular hydrogen bonding in spite of its negative charge at physiological pH. When PA is completely ionized at high pH, the I_{pi} drops appreciably (Blume and Eibl, 1979; Eibl and Blume, 1979, Eibl, 1983), probably due to increased charge repulsion and loss of hydrogen bonding interactions between the PA headgroups.

Sphingolipids posses additional chemical groups, which are not found in phospholipids, that may also participate in inter-molecular hydrogen bonding. The amido proton and the C-3-hydroxyl group of the

ceramide moiety, as well as the 2'-hydroxyl groups present on a significant portion of sphingolipid N-acyl chains, may all serve as hydrogen-bond donors. As well, the glycosphingolipids (cerebrosides, sulfatides, and gangliosides) possess multiple potential hydrogen bonding moieties (hydroxyl and amide groups) in their headgroups which have a large effect on their phase behaviour.

The transition temperatures of various sphingomyelins and synthetic sphingomyelin analogues have typically been found to be only slightly elevated above those of phosphatidylcholines of similar chain lengths (Barenholz et al., 1976; Barenholz and Thompson, 1980; Barenholz and Gatt, 1982; Curatolo et al., 1982, 1985). However, pure synthetic sphingomyelins can exhibit complex gel-phase behaviour, forming stable gel states with considerably greater enthalpies and entropies of chain melting than are observed for the corresponding phosphatidylcholines (Barenholz et al., 1976, Cohen et al., 1984, Curatolo et al., 1982, 1985). It is thought that this distinctive physical behaviour of the sphingomyelins may be due to intermolecular hydrogen bonding between sphingomyelins that is not possible for phosphatidylcholines (Curatolo et al., 1982, 1985).

The simple glycosphingolipids galactosyl cerebroside and sulfatide exhibit relatively high transition temperatures when compared to phospholipids or sphingomyelins with similar acyl chains. It has been suggested that this behaviour reflects the occurrence of strong intermolecular hydrogen bonding between glycosphingolipid molecules in the gel phase, thus stabilizing this phase relative to the liquid-crystalline phase (Boggs et al., 1984). This concept is supported by the finding that synthetic glycolipids consisting of a sugar residue linked to the C-1 position of a 2,3-ditetradecyl-sn-glycerol, were found to posses T_M's 17-27°C higher than their corresponding ditetradecyl PC's (Hinz et al., 1985). Studies of the phase behaviour of sulfatides (Boggs et al., 1984, 1985) and cerebrosides (Clowes et al., 1971; Bunow, 1979; Bunow and Levin, 1980; Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982; Curatolo and Jungalwala, 1985; Reed and Shipley, 1987) with different N-acyl chains have shown that the transition temperatures and

enthalpies of these species do not depend strongly on the acyl chain length. This result suggests that hydrogen-bonding interactions between polar headgroups have a greater influence on the thermotropic behaviour of these lipids than do the van der Waals' interactions between the hydrocarbon chains. Synthetic sulfatide species exhibit lower T_M's than do the corresponding cerebrosides, probably reflecting the presence of electrostatic repulsions between the sulfogalactosyl headgroups (Boggs et al., 1984).

The crystal structure of a synthetic cerebroside has been reported by Pascher and Sundell (1977). Within the crystal structure, the planes of the sugar rings lie almost parallel to the plane of the bilayer, and the sugar headgroups form an extensive lateral network of hydrogen bonds. Similar headgroup interactions have been indicated in ^2H NMR studies of N-16:0 cerebrosides (Skarjune and Oldfield, 1979). The sphingosine hydroxyl groups and the amide-linked hydroxy fatty acids in these systems are also involved in lateral hydrogen-bonding interactions. Such interactions are probably important in the formation of highly stable, high-melting gel phases by cerebrosides (Boggs, 1987; Curatolo, 1987)

1.3.2.2 Binary Lipid Mixtures

Although studies involving pure lipid species have indicated that lipids with different headgroup structures can exhibit quite different physical behaviour, this finding is of little biological relevance if these distinct behaviours are not reflected in the properties of membranes with mixed lipid composition. Experiments involving binary mixtures of lipids with different headgroups have been extremely important to address this and other questions concerning the roles of lipids with different headgroups in determining the physical properties of biological membranes.

Early experiments studying the phase equilibria of binary mixtures of symmetric phospholipid species of similar chain length have generally indicated limited solid-phase miscibility and regular, though not ideal, mixing of the lipid species in the liquid-crystal-

line state. Most PE/PC systems with symmetric acyl chains have shown partial immiscibility in the gel-state and complete, but apparently non-ideal mixing in the liquid-crystalline state (Wu and McConnell, 1975; Luna and McConnell, 1978; Blume et al., 1982). Mixtures of DPPE with DEPC were originally reported to exhibit liquid-liquid immiscibility (Wu and McConnell, 1975), although more recent studies employing DSC (Silvius, 1986, Brauner and Mendelsohn, 1986) and FT-IR (Brauner and Mendelsohn, 1986) have failed to confirm this finding. It has been suggested that previous analyses of lipid miscibility in PE/PC systems have often failed to account adequately for the solid-phase polymorphism exhibited by the PC component (Silvius, 1986), which may lead to erroneous conclusions concerning the thermodynamics of lipid mixing in the liquid-crystalline as well as the gel-state.

Several studies of the mixing behaviour of phosphatidylcholines with phosphatidylserines or phosphatidic acids (Shimshick and McConnell, 1973; van Dyck et al. 1977, 1978; Luna and McConnell, 1977, 1978; Stewart et al., 1979; Hui et al., 1983; Silvius and Gagné, 1984a) have generally indicated high miscibility in the liquid crystalline state, although limited miscibility of these species in the gel-state of saturated PC/PS and PC/PA mixtures has been reported (Luna and McConnell, 1977; van Dijck et al., 1978; Silvius and Gagné, 1984a). Silvius and Gagné (1984a) have suggested that conflicting results obtained in earlier studies could be due to incomplete sample equilibration.

DSC experiments involving binary mixtures of PE and PS species (Silvius and Gagné, 1984b) and of PE and PA species (Graham et al., 1986) of like-acyl composition have indicated that such systems exhibit near-ideal miscibility in both the gel and liquid-crystalline states.

Lateral segregation of anionic from neutral lipids has been reported in a number of binary lipid mixtures in the presence of divalent cations, particularly calcium (PC/PA: Ohnishi and Ito, 1974; Galla and Sackmann, 1975; Jacobson and Papahadjopoulos, 1975; Hartmann et al., 1977; van Dijck et al., 1978; Verkleij et al., 1982; Caffrey and Feigenson, 1984; Graham et al., 1985; Kouaouci et al., 1985;

Haverstick and Gaser, 1987; Leventis et al., 1987; PE/PA: Graham et al., 1985; PC/PS: Ohnishi and Ito, 1974; Tokutomi et al., 1981; Ohki et al., 1981; Hoekstra, 1982; Hui et al., 1983; Florine and Feigenson, 1987; Feigenson, 1989; PE/PS: Cullis and Verkleij, 1979; Tokutomi et al., 1981; Tilcock and Cullis, 1981; Silvius and Gagné, 1984b). Such phase separations are usually of the solid/liquid-crystalline type, not the liquid-crystalline/liquid-crystalline type.

Calorimetric studies of phosphatidylcholine/sphingomyelin mixtures (Untract and Shipley, 1977; Barenholz et al., 1976) have shown these systems to be completely miscible at physiological temperature, although lateral separation of sphingomyelin-rich phases occurs upon cooling. Steady-state fluorescence techniques and freeze fracture microscopy have shown that phosphatidylcholine/sphingomyelin vesicles (Calhoun and Shipley, 1979; Lentz et al., 1981) exhibit thermotropic behaviour similar to that of DMPC-DPPC mixtures (Shimschick and McConnell, 1973; Chapman et al., 1974; Lee, 1975), except that the former system exhibits gel state immiscibility at low temperatures. Lentz and coworkers (1981) have suggested that differences in acyl chain composition, and not in head group structure, comprise the major factor in determining the phase separations in sphingomyelin/PC systems.

The mixing of cerebrosides with phospholipids has been studied by a variety of techniques to date (Bunow and Levin, 1980; Skarjune and Oldfield, 1982; Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986; Bunow and Levin, 1988; Johnston and Chapman, 1988; Koynova et al., 1988). Such systems generally exhibit almost complete immiscibility in the gel phase. At physiological temperatures, a homogeneous liquid-crystalline phase is formed only at relatively low mole fractions of cerebroside. Above this 'solubility limit', there is separation of a cerebroside-rich gel phase. This behaviour is believed to be due to the presence of strong hydrogen-bonding interactions between the cerebroside headgroups. Rintoul and Welti (1989) have recently shown in sulfatide/DEPC mixtures that sulfatide exhibits somewhat better miscibility with PC at physiological temperatures than the corresponding cerebroside. It is

important to note that the glycosphingolipid/PC systems just discussed exhibit gel/liquid-crystalline, and not liquid-crystalline/liquid-crystalline phase separations.

In summary, a variety of binary phospho-and sphingolipid mixtures have been found to exhibit at least partial immiscibility in the gel state and appear to mix in a nonideal manner in the liquid-crystalline state. Clear evidence for liquid-crystalline/liquid-crystalline phase separations has not been reported to date in mixtures of lipid species with similar acyl chain lengths, although both 'clustering' and gel/liquid-crystalline phase separations may be possible for some lipid mixtures of this type under physiological conditions.

1.3.3 The Effect of Acyl Chain Structure on Lipid-Lipid Interactions:

Given that the hydrocarbon chains of a lipid molecule closely interact with neighbouring hydrocarbon chains in the interior of a bilayer, it is not surprising that changes in the length and degree of unsaturation of these chains can have a great effect on membrane behaviour. The C-1 acyl chains of the phospholipids present in most eukaryotic systems are typically saturated, while the C-2 chains are typically unsaturated (Holub and Kuksis, 1978). Most lipid mixing studies to date have dealt with 'symmetric chain' phospholipids, whose two acyl chains are either chemically identical or (in a slightly looser definition) very similar in length.

1.3.3.1 Lipids with Identical Acyl Chains:

While lipids bearing two identical acyl chains (which I will refer to below as 'like-chain' species) are minority species in most biological systems, studies of such lipid species have revealed a number of important general trends regarding the relationship of lipid acyl chain structure to the properties of the lipid bilayer.

A number of groups have shown that for a given series of like-chain lipids with the same headgroup and degree of unsaturation, the enthalpies and entropies of the gel-to-liquid-crystalline transitions increase as the acyl chain length increases (Ladbrooke and Chapman, 1969; Lee, 1975; Mabrey and Sturtevant, 1976, 1978; Huang et al., 1982; Silvius, 1982; Huang and Levin, 1983). The explanation of this trend is as follows: as one increases the acyl chain length, the attractive van der Waals' forces between adjacent lipid acyl chains increase, and hence more energy is required to weaken these interactions on melting from the gel to the liquid-crystalline state. Studies of binary mixtures of saturated like-chain phosphatidylcholines of differing acyl chain lengths have shown that in cases where the difference in acyl chain lengths is slight, mixing is nearly ideal. This has been demonstrated for mixtures of DMPC with DPPC (Shimshick and McConnell, 1973, Chapman et al., 1974, Lee, 1975), dielaidoyl phosphatidylcholine (DEPC) with DPPC (Wu and McConnell, 1975), and DPPC with distearoyl phosphatidylcholine (DSPC) (Shimshick and McConnell, 1973, Matubayasi et al., 1986). Mabrey and Sturtevant (1976) have shown that binary mixtures of like-chain phosphatidylcholines whose acyl moieties differ by 6 or more carbons exhibit full (but not ideal) miscibility in the liquid crystalline state, yet undergo lateral phase separation in the gel state. Such a result is not unexpected, since mixing of such disparate species in the gel phase would involve extremely mismatched pairing of acyl chains in the centre of the bilayer, an energetically unfavourable state.

Unsaturated like-chain phospholipids have generally been shown to have a decreased gel-to-liquid-crystalline transition temperature when compared to their saturated counterparts, particularly when their cis-double bonds are located near the centers of the acyl chains (Barton and Gunstone, 1975). This fact, coupled with the observation that most phospholipids possess one unsaturated acyl chain, has led to the general belief that unsaturated fatty acids have an extremely important role in maintaining the fluidity (ie. the

liquid-crystalline state) of biological membranes at physiological temperatures.

Mixtures of saturated and unsaturated like-chain lipids with the same headgroup typically show lower miscibility, at least in the gel state, than do binary mixtures of saturated like-chain lipids with similar acyl chain lengths (for a comprehensive listing of the miscibilities of various binary phospholipid mixtures, see Silvius, 1982). Thus, the saturated/unsaturated mixed-acyl phospholipids present in biological membranes, have the added advantage of avoiding the low miscibility that occurs with mixtures of disaturated and di-unsaturated like-chain lipids.

Despite their biological importance, relatively few studies have been undertaken involving the thermotropic behaviour of saturated/unsaturated phospholipids. These studies have shown that mixed chain phospholipid species typically show broad, uncooperative phase transitions, likely due to the disruptive effect of the saturated chain on the formation of the gel phase (Keough and Davis, 1980; Davis et al., 1981; Epand and Bottega, 1988).

1.3.3.2 Lipids With Asymmetric Acyl Chains:

In recent years, a number of studies have been performed on asymmetric phospholipids (which I define as lipids bearing acyl chains of significantly different lengths). Such studies are important, as a number of natural lipids have structures of this type. For example, more than 50% of sphingomyelins found in nervous tissue have either saturated or unsaturated N-acyl chains of longer than 18 carbons (Barenholz and Thompson, 1980). Similar fatty acyl compositions are found in naturally occurring cerebrosides (Abrahamsson et al., 1977), sulfatides (Haines, 1971) and gangliosides (Yu and Ando, 1980).

Several groups have systematically studied the properties of dispersions of saturated phosphatidylcholines in which the discrepancy between the lengths of the two acyl chains is progressively increased (Keough and Davis, 1979; Stümpel et al., 1981; Chen and

Sturtevant, 1981). These studies have revealed that the physical behaviour of such lipids is dependent on both the difference in chain length and the position in which the asymmetric chains are attached to the glycerol backbone. As one might expect, the T_M's of mixed-acyl phosphatidylcholines are generally found to lie between the T_M's of the two corresponding like-chain PCs (Keough and Davis, 1979).

An interesting feature of the behaviour of some asymmetric phospholipids was first observed by Tardieu et al. (1973) in an X-ray study of the crystal phase of C(18):C(10) PC at 2-4% hydration. These workers found that under these conditions the thickness of bilayers composed of this lipid was identical to that of DMPC. It was suggested that this was brought about through interdigitation of the acyl chains in the centre of the bilayer i.e., the stearoyl chains belonging to the PC molecules in one leaflet paired tail-to-tail with the shorter decanoyl chains of the PC's in the opposite leaflet.

These earlier studies were sometimes complicated by difficulties in obtaining lipids with unlike acyl chains in high isomeric purity. More recent studies involving more pure lipid preparations have generally upheld the major findings of earlier work. Preparations of mixed-acyl phospholipids of high isomeric purity (e.g. by the method of Mason et al., 1981a) have enabled the more exact analysis of these lipids by a number of physical techniques, including high resolution DSC (Mason et al., 1981b; Xu et al., 1987; Mason, 1988) X-ray diffraction (Serrallach et al., 1984; McIntosh et al. 1984; Hui et al., 1984; Mattai et al., 1987; Xu and Huang, 1987), Raman (Huang et al., 1983), ^{31}P NMR (Xu et al., 1987), and ESR (Boggs and Mason, 1986). To date, most of this work has focused on the properties of asymmetric phosphatidylcholines. The physical behaviour of these species can be rationalized by considering the effect of the chain-length inequivalence on the packing of the hydrocarbon chains within the interior of the bilayer (Mason et al. 1981b). In a recent review, Huang and Mason (1986) have classified asymmetric phosphatidylcholines with respect to the type of interdigitated bilayer formed in the hydrated gel state. Such phases can be of three types: fully interdigitated, mixed interdigitated, and partially inter-

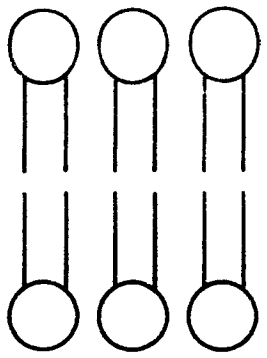
digitated (see Figure 2). Which interdigitated phase an asymmetric lipid will adopt can be predicted from the magnitude of its 'chain-length inequivalence parameter' ($\Delta C/CL$), which is defined as the ratio of the absolute difference in chain length of the two hydrocarbon chains divided by the length of the longer chain. Highly asymmetric phospholipids (e.g. C(18):C(0)-PC, C(18).C(2)-PC) with a $\Delta C/CL$ value of approximately 1.0 form fully interdigitated phases, in which the long acyl chain spans fully the hydrocarbon portion of the bilayer and the short chain (if one is present) runs parallel to the bilayer surface. As the difference in acyl chain length decreases to $\Delta C/CL$ values of 0.6 to 0.4 (e.g., for C(18):C(10)-PC), the lipids adopt a mixed interdigitated arrangement. In such a bilayer, the shorter chain in one leaflet is packed end-to-end with the shorter chain of a lipid in the other leaflet, while the long chains of the lipids fully span the bilayer. Partially interdigitated bilayers are formed by asymmetric lipids whose two chains are more similar in length (e.g. C(18):C(12)-PC, C(18):C(16)-PC, or C(16):C(14)-PC). In this state, the short acyl chains pack end to end with the longer acyl chains from the opposite leaflet.

Mason (1988) has determined phase diagrams for binary mixtures of DSPC and a series of phosphatidylcholine species with the structure C(18):C(n) where n = 18, 16, 14, 12, and 10. He noted that as the degree of acyl chain asymmetry of the latter species increased, so did its tendency to form an interdigitated gel phase that segregated laterally from non-interdigitated DSPC-rich gel state domains. Perhaps the most interesting of the mixtures described in this paper is the binary system DSPC/C(18):C(10)-PC, which exhibits partial immiscibility in both the gel and liquid-crystalline phases. The observation of partial immiscibility of these two species in the liquid-crystalline state suggests that highly asymmetric polar lipids could form segregated domains (gel or even liquid-crystalline) in biological membranes, in which they are combined with lipids with more nearly symmetric acyl chains.

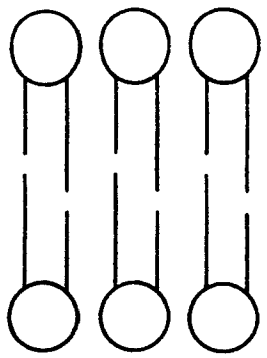
Relatively few studies to date have examined the effect of hydrocarbon chain asymmetry on the phase behaviour of sphingolipids

Figure 2.

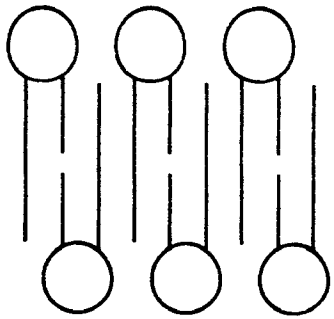
Schematic figure of possible bilayer structures. Two interdigitated phases as defined by Huang and Mason (1986) are represented. The fully interdigitated phase is not shown. The lipid polar headgroups are represented as circles and the acyl chains as lines. For complete descriptions of these phases, see text.



NONINTERDIGITATED



PARTIALLY
INTERDIGITATED



MIXED
INTERDIGITATED

In a Raman spectroscopic study of aqueous dispersions of N-lignoceroyl sphingomyelin, Levin et al. (1985) observed two thermal phase transitions at 48.5° and 54.5°C. These researchers proposed a model in which the lower transition is ascribed to the conversion of a mixed interdigitated gel state to a partially interdigitated gel state, while the higher corresponds to conversion from the partially interdigitated gel state to a liquid- crystalline phase. X-ray scattering experiments on sphingomyelin vesicles also suggest chain interdigitation when the length of the N-acyl chain significantly exceeds the length of the sphingosine hydrocarbon chain (Maulik et al., 1986).

Sulfatides with long N-acyl chains (24 to 26 carbons) are believed to form interdigitated phases (Boggs et al., 1988). A spin labelled fatty acid, when incorporated into bilayers of sulfatides bearing long, but not shorter N-acyl chains (16 and 18 carbons), showed a high degree of motional restriction similar to that which occurs in the mixed interdigitated bilayers formed by highly asymmetric PC species (Boggs and Rangaraj, 1985, Boggs and Mason, 1986).

In a recent ESR study, Grant and coworkers found that low mole fractions (2 mol%) of a long-chain (N-24.0) spin-labeled lactosyl ceramide species did not form a discrete phase in phospholipid bilayers, and instead appeared to be accommodated into the bilayer by interdigitating into the opposite monolayer (Grant et al., 1987; Mehlhorn et al., 1988). Evidence suggesting such acyl chain interdigitation was not found for a shorter-chain (N-16:0) species.

Calorimetric studies on synthetic galactosylceramides have demonstrated the existence of a partially interdigitated phase for the N-24:0 species (Reed and Shipley, 1987). Other studies examining the thermotropic behaviour of natural cerebrosides have shown that such systems characteristically exhibit complex solid-phase polymorphism which could be partially due to the formation of such a phase (Bunow, 1979, Freire et al., 1980, Curatolo and Jungalwala, 1985).

1.3.4 Phospho- and Sphingolipid Mixing With Cholesterol:

The literature dealing with the effects of cholesterol on the physical behaviour of both natural and artificial membranes is vast, reflecting the extremely important role of cholesterol in biological systems. Cholesterol has been shown to have a variety of unique effects on lipid bilayers, some of which are discussed below.

One of the important properties of cholesterol is its ability to affect membrane ordering. ^{1}H NMR studies (Oldfield et al, 1978) of deuterium-labelled phospholipids in bilayers have shown that there are different degrees of motional order (measured as the 'order parameter') associated with different segments of the lipid hydrocarbon chains. The order parameter for the portion of the chain nearest the glycerol backbone is high and nearly constant from the C-2 to the C-10 methylene groups. As one examines the ordering of methylene segments in the 'distal' half of the acyl chain approaching the centre of the bilayer, a gradual decrease in the order parameter is observed, such that the movement of the terminal methyl groups is nearly isotropic. When cholesterol is incorporated into the bilayer, it increases further the motional order of the portions of the acyl chains near the glycerol backbone while having little effect on chain ordering near the bilayer centre (Stockton and Smith, 1976). This effect is different from the increase in motional order experienced by all methylene segments along the hydrocarbon chain when a bilayer enters the gel state (Yeagle, 1985). Cholesterol has an overall condensing effect on liquid-crystalline lipid bilayers, resulting in a decrease in the surface area per phospholipid molecule.

Another well-studied effect of cholesterol in lipid bilayers is its ability to alter substantially the enthalpy and the cooperativity of gel- to-liquid-crystalline phase transitions. Estep and coworkers (1978) showed that 20 - 25 mol% cholesterol incorporated into DPPC bilayers caused the elimination of the sharp gel-to-liquid-crystal phase transition. Mabrey et. al. (1978) and Davis and Keough (1983) reported similar effects of cholesterol on the phase transitions of DMPC and mixed-chain phospholipids, respectively. Employing X-ray

diffraction methods, Hui and He (1983) found that bulk lateral phase separations of the phospholipid and sterol components do not appear to occur in these mixtures at temperatures about the main phase transition of the phospholipid component. These researchers have suggested instead that at these temperatures, there may be lateral segregation into cholesterol-rich and cholesterol-poor 'micro-domains' rather than bulk phases of distinct compositions. A number of groups employing a variety of techniques, including NMR (Blume and Griffin, 1982), ESR (Presti and Chan, 1982; Recktenwald and McConnell, 1981), and fluorescence (Lentz et al., 1980), have presented data consistent with the occurience of bulk phase separation into cholesterol 'rich' and 'poor' domains at temperatures well below the main temperature transition of the phospholipid species.

The effects of cholesterol on the properties of sphingolipid bilayers have also been studied in some detail. Estep et al. (1981) have examined the effects of cholesterol addition to N-18:0 sphingomyelin bilayers. Similar to the results obtained for DPPC/cholesterol mixtures, addition of up to 20 mol % cholesterol resulted in the broadening and eventual disappearance of the sharp 56-57 °C main transition peak of N-18:0 sphingomyelin. Below the main temperature transition of the sphingomyelin, complex solid phase behaviour was observed, consistent with the formation of cholesterol-rich and cholesterol-poor domains.

Ruocco and Shipley (1984) have extensively studied the mixing of cholesterol with N-16:0 galactosylsphingosine by differential scanning calorimetry and X-ray diffraction. With increasing cholesterol up to 50 mol%, the cerebroside gel-to-liquid-crystalline peak decreased in enthalpy. The X-ray data indicates that a solid cerebroside phase and a solid cholesterol phase are present at temperatures below the main transition temperature of the cerebroside. These researchers suggest that this low temperature lipid-lipid immiscibility results from preferential cerebroside-cerebroside associations via hydrogen bonding.

The ability of cholesterol to alter phospho- and sphingolipid phase transitions has been exploited to suggest the existence of

specific cholesterol-lipid associations. Van Dijck et al. (1976) utilized liposomes composed of 1:1 mixtures of different PE species to demonstrate that cholesterol addition to this system specifically decreased the enthalpy of the lower-melting PE, suggesting a preferential association of cholesterol with that component. Interestingly, however, in 1:1 mixtures of PE and PC, cholesterol preferentially abolished the transition of the PC component, regardless of whether the PC was the higher or lower melting component. This result suggested a preferential interaction of cholesterol with PC 'in competition' with PE. Further calorimetric studies utilizing the same basic technique (Demel et al., 1977; van Dijck, 1979), as well as experiments involving the partitioning of radiolabelled cholesterol between different lipid environments (Yeagle and Young, 1986) have enabled the construction of the following relative ranking of cholesterol affinity for different lipids: sphingomyelin > PS > PG > PC > PE (for a contrary report utilizing the cholesterol partitioning technique, see Lange et al., 1979).

1.3.5 The Implications of Lipid Clustering in Natural Membranes:

As stated earlier in this chapter, putative lipid 'domains' in liquid-crystalline bilayers have been suggested to play important roles in several membrane-mediated phenomena. Several laboratories have presented evidence suggesting the existence of such phases in natural membranes, usually on the basis of rather indirect, if suggestive evidence (Dragsten et al., 1981; Spiegel et al., 1985; Metcalf et al., 1986; van Meer and Simons, 1986; Gordon et al., 1987; Yechiel and Edidin, 1987). The following discussion will concentrate on the clustering behaviour of glycosphingolipids, as such species are believed to be more likely to form such phases than are naturally occurring phospholipids.

Tillack and coworkers (1982) have shown that asialogangliosides form clustered domains in DMPC bilayers at relatively low mole percentages. This has interesting implications, since small clusters

of gangliosides, and possibly other glycolipids, have been shown to act as multivalent membrane receptors and surface antigens (Cuatrecasas, 1973; Haywood, 1974; Revesz and Greaves, 1975; Mullin et al., 1976; Holmgren et al., 1980; Critchley et al., 1981). The most studied system of this type is the binding of the B subunit of cholera toxin to G_{M1}. The B subunit possesses multiple G_{M1} binding sites. Two possible mechanisms of B subunit binding to G_{M1} have been suggested; the subunit may bind to a single G_{M1} molecule and then laterally diffuse as a receptor-ligand complex until it binds other receptors (Saffman and Delbruck, 1975), or G_{M1} clustering may be a precondition to B binding. Lateral redistribution of gangliosides upon toxin binding has been observed using fluorescent ganglioside probes, (Sedlacek et al., 1976, Reed et al., 1987) suggesting the first mechanism may be the more valid of the two. Recently, however, Masserini and coworkers (1988) have shown in an analogous system that V. cholerae sialidase recognizes G_{D1a} 'micro-domains' on membrane surfaces.

Cerebrosides have been shown to have large effects on phospholipid membrane morphology (Correa-Freire et al., 1979, Curatolo and Neuringer, 1986). It has been suggested that in natural membranes where these lipids are found in high concentrations, cerebrosides can affect membrane shape, possibly through specific intermolecular interactions, and may be involved in the mechanism of formation of membranes with unusual morphology (Curatolo, 1987). While the clustering behaviour of glycosphingolipids has been suggested to be due to intermolecular hydrogen bonding between the carbohydrate residues of adjacent polar headgroups (Boggs, 1987), it is possible that acyl chain asymmetry may be involved as well, as naturally-occurring glycolipid species are composed of a large percentage of long N-acyl chains.

Acyl chain asymmetry may provide a mechanism for information transfer across cell membranes through the formation of interdigitated phases. Several studies have shown that asymmetric sphingolipids can couple the outer and inner membrane leaflets through interdigitation of their acyl chains in the centre of the

bilayer. A cluster of such lipids on one side of the bilayer could perturb acyl chain (and even headgroup) packing in the opposite bilayer leaflet (Nicholson, 1976; Schmidt et al., 1978; Levin et al., 1985; Grant et al., 1987; Boggs et al., 1988; Melhorn et al., 1988; Reed and Shipley, 1987).

1.4 Introduction to the Following Chapters:

From the preceding pages, it should be evident that while a great deal of work has been performed in attempt to elucidate the functional and structural roles of membrane lipids, there are several questions that have only recently begun to be addressed. The suggestion that differential nonideal interactions between different lipids can thermodynamically favour the formation of lipid 'clusters' or even discrete lipid phases in liquid-crystalline bilayers, while interesting, is as yet unsubstantiated.

As stated in the preface of this chapter, the objective of my thesis is to examine how specific components of lipid structure i.e. headgroup and acyl chain length 'mismatch', affect lipid-lipid interactions in model systems. This objective will be approached using two different experimental techniques; fluorescence spectroscopy and high-sensitivity differential scanning calorimetry.

Chapter 2 will examine how headgroup structure affects the equilibrium partitioning of fluorescent phospho- and sphingolipid probes between different lipid bilayer environments. With this technique, probes are allowed to partition between two populations of vesicles composed of different lipid species, in this case, between those composed of phospholipids with hydrogen-bonding capability vs. those containing non-hydrogen bonding phospholipids and between cholesterol and cholesterol-free phospholipid bilayers. From the equilibrium concentration of probe in the two vesicle populations, one can determine the relative energies of different types of headgroup-headgroup interactions.

Chapter 3 will describe the thermotropic behaviour of binary mixtures of DPPC with phosphatidylcholine, sulfatide, and cerebroside

species in which one hydrocarbon chain is at least 11 carbons longer than the other. The purpose of these experiments is to determine whether asymmetric sphingolipids are more prone to segregate from a symmetric phosphatidylcholine than are sphingolipids with nearly symmetric hydrocarbon chains, whose miscibilities with symmetric-chain phospholipids have been studied previously.

CHAPTER TWO:

Partitioning of Exchangeable Fluorescent Phospholipids and Sphingolipids Between Different Lipid Bilayer Environments

2.1 Abstract[1]:

Exchangeable phospho- and sphingolipid probes (phosphatidylcholine, -ethanolamine, -serine, and -glycerol, phosphatidic acid, sphingomyelin, cerebroside and sulfatide) have been synthesized in which one acyl chain is substituted with a fluorescent bimanyl, (7-dimethylaminocoumarin-3-yl)-, or diphenylhexatrienyl group. The distribution of these probes between two different populations of lipid vesicles can be readily monitored by fluorescence intensity measurements, as described by Nichols and Pagano (Biochemistry 21 [1982], 1720-26), when one of the vesicle populations contains a low mole fraction of a non-exchangeable quencher, (12-DABS)-18 PC. Probes with different polar headgroups were found to differ only modestly in their relative affinities for vesicles composed of 'hydrogen-bonding' lipids (defined in the present work as those that can both donate and accept hydrogen bonds e.g. PE and PS) vs. 'non-hydrogen-bonding' lipids (those that can only accept hydrogen bonds e.g. PC, PG, or O-methyl PA). Probes with different headgroups also show modest, albeit reproducible differences in their relative affinities for cholesterol-containing vs. cholesterol-free PC/PC vesicles. The results suggest that lipids with different headgroup structures may mix more nearly ideally in liquid-crystalline lipid bilayers than would be predicted from previous analyses of the phase diagrams for binary lipid mixtures.

---

[1]The material presented in this chapter has been published as part of Gaidam, M.A., Itovitch, J.J., and Silvius, J.R., (1989) Biochemistry 29, 884-893.

2.2 Introduction:

The nature of the lateral interactions between different lipid species can strongly influence the physical properties and the organization of bilayer membranes that contain multiple lipid components. Because of this fact, numerous studies have examined the interactions between different types of polar lipids in bilayers, using a variety of physical techniques (for reviews, see Lee, 1977; Gaffney and Chen, 1977; Mabrey and Sturtevant, 1978; Melchior and Steim, 1979; McElhaney, 1984; Keough and Davis, 1984; Thompson and Tillack, 1985; Brauner and Mendelsohn, 1986; Curatolo, 1987). To date, most thermodynamic information regarding the interactions between different lipids in bilayers has been derived from analyses of the phase diagrams for various binary mixtures of phospho- and sphingolipids. The principles of regular solution theory are often applied in such analyses to estimate quantitatively how differences in the headgroups and/or the acyl chains of different lipid species affect their free energy of mixing (Lee, 1977; van Dijck et al., 1977; Von Dreele, 1978; Cheng, 1980; Keough and Davis, 1984).

The above approach to examine the thermodynamics of lipid mixing in bilayer membranes suffers from several significant limitations, particularly for the analysis of systems more complex than binary mixtures, or for the study of lipid mixtures that exhibit complex solid-phase polymorphism. Alternative approaches can be useful to provide information about the thermodynamics of mixing between different lipid species in such systems, for which a full characterization of the phase diagram is not desirable or feasible. One such alternative approach is the measurement of the equilibrium distribution of exchangeable lipid species between different types of lipid vesicles, a method that can detect differences in the free energy of the exchangeable species in different lipid environments. This approach has been exploited previously to examine the equilibrium partitioning of radiolabeled cholesterol (Lange et al., 1979; Wattenberg et al., 1983; Fugler et al., 1985; Rujanavech and Silbert, 1986; Yeagle and Young, 1986) and of fluorescent phosphatidylcholine

and phosphatidic acid probes (Nichols and Pagano, 1982) between different types of lipid vesicles.

In the present study, the resonance energy transfer-based method described by Nichols and Pagano (1982) has been adapted to examine the partitioning of a variety of exchangeable fluorescent lipids between lipid vesicles of various compositions. This method requires no physical separation of different vesicle populations; instead the equilibrium distribution of a fluorescent probe between two populations of vesicles is made readily measurable by including in one vesicle population a nonexchangeable quencher of the probe fluorescence. Using this approach, it is possible to evaluate how the structure of the polar headgroup of a lipid molecule affects its partitioning between different lipid bilayer environments, which vary in their lipid polar headgroup compositions and/or sterol contents.

2.3 Materials and Methods:

2.3.1 Materials:

1-Palmitoyl-2-(8-(bimanylmercapto)octanoyl)- phosphatidylcholine ([8-bimane]-PC), and the corresponding species with an 11-bimanylmercaptoundecanoyl chain at the 2-position ([11-bimane]-PC), were synthesized as described previously for the synthesis of the latter compound (Silvius et al., 1987) The intermediate phosphatidylcholine species in the above syntheses were in all cases purified by preparative thin-layer chromatography. 1-Palmitoyl-2-(11-((7-dimethylamino-coumarin-3-yl)carbonyl)methylaminoundecanoyl)-phosphatidylcholine ([11-DMCA]-PC) was prepared by labeling 1-palmitoyl-2-(11-(methylamino)undecanoyl)-phosphatidylcholine, synthesized as described previously (Silvius et al., 1987), with a slight excess of 7-dimethylaminocoumarin-3-carboxylic acid N-hydroxysuccinimide ester (Molecular Probes, Junction City, Ore.) in 8:2:0.1 (vol/vol/vol) $CHCl_3/CH_3OH$/triethylamine for 6 hours at 25 °C. The labeled phosphatidylcholine was purified by preparative thin-layer chromatography, using 65:35:2.5:2.5 (vol/vol/vol/vol) $CHCl_3/CH_3OH/H_2O$/conc. NH_4OH as the developing solvent. 1-Decanoyl-2-(3-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)propanoyl)-sn-glycero-3-phosphocholine was prepared by acylation of 1-decanoyl-lyso-phosphatidylcholine with the anhydride of (3-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)propanoic acid in the presence of 4-pyrrolidinopyridine (Mason et al., 1981)

The fluorescent phosphatidylcholines from the above preparations were converted to the corresponding PE, PS, PG and PA derivatives by phospholipase D-catalyzed transphosphatidylation or hydrolysis, as described by Comfurius and Zwaal (1977). Efficient transphosphatidylation of these probes could be achieved using incubation times or phospholipase concentrations that were ten- to forty-fold less than those required for the transphosphatidylation of longer-chain PC's. The transphosphatidylated probes were extracted from the reaction mixtures and purified by preparative thin-layer chromatography.

N-(11-bimanylmercapto)undecanoyl-sphingomyelin and -cerebroside were prepared by coupling sphingosinephosphorylcholine (Cohen et al., 1984) or galactosylpsychosine (Radin, 1974, as modified by Koshy and Boggs, 1982) to 11-S-(mercaptomethyl)mercaptoundecanoic acid for 6 hr. at 37° C in the presence of 2,2'-bispyridyldisulfide and triphenylphosphine (Kishimoto, 1975). The intermediate N-(11-S-(mercaptomethyl)mercapto)undecanoyl- sphingolipids from the above reactions were deprotected with excess dithiothreitol and labeled with a slight excess of monobromobimane (Silvius et al., 1987). N-(11-bimanylmercapto)undecanoyl-sulfatide was prepared in a similar manner from sulfogalactosylpsychosine (Koshy and Boggs, 1982), but the acylation step was carried out using the acyl chloride derivative of 11-S-(mercaptomethyl)mercaptoundecanoic acid (Koshy and Boggs, 1983). All of these sphingolipid probes, as well as the intermediate protected mercaptoacyl compounds, were purified by preparative thin layer chromatography (developing solvents: 65:25:4 $CHCl_3/CH_3OH$/conc. NH_4OH for cerebroside, 80:20:5:3 $CHCl_3/CH_3OH/CH_3COOH/H_2O$ for sulfatide and 50:40:10:6 $CHCl_3/CH_3OH/CH_3COOH/H_2O$ for sphingomyelin). All fluorescent labeling reactions were carried out with exclusion of light and under nitrogen.

Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, Ala.), and phosphatidylethanolamine and phosphatidylglycerol were prepared from it by enzymatic transphosphatidylation (Comfurius and Zwaal, 1977). Dioleoyl phosphatidylserine (DOPS) and 1,2-dioleoyloxy-3-(trimethylammonium)propane (DOTAP) were synthesized as described previously (Silvius and Gagné, 1984; Stamatatos et al., 1988). Cholesterol was obtained from Nu-Chek Prep (Elysian, Minn.) and was twice recrystallized from ethanol. All common inorganic chemicals were of reagent grade or better. All organic solvents were redistilled before use; diethyl ether used for vesicle preparations was redistilled, stabilized with 1% water and stored shielded from light at 4 °C.

2.3.2 Methods:

Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation and filtered through 0.1 um pore size Nucleopore membranes (Wilschut et al., 1980). The buffer used to prepare the vesicles was 150 mM NaCl, 2 mM Tes, 0.2 mM EDTA, pH 7.4. Measurements of the trapped volumes of such vesicles, carried out using carboxyfluorescein as an internal marker (Wilschut et al. 1980), gave values of 3-4 ul per umole lipid, consistent with average vesicle diameters of ca. 850-1150 A in these preparations.

Vesicles were labeled with exchangeable lipid probes using either of two protocols. 'Symmetrically' labeled vesicles were prepared by including 0.5-2 mole% of the fluorescent probe into the lipid mixture dispensed for the initial preparation of vesicles. 'Asymmetrically' labeled vesicles were prepared by incubating preformed, unlabeled vesicles with sonicated dispersions of the probe in buffer, normally at vesicle and probe concentrations of 1 mM and 5-20 uM, respectively, for 1-2 hr at 37° C.

The partitioning of exchangeable lipid probes between different types of lipid vesicles was measured by incubating a fixed amount of probe-labeled donor vesicles (7.5-15 nmoles) with varying amounts of a second, unlabeled population of acceptor vesicles (0-300 nmoles) in a total volume of 3 ml. One of the two vesicle populations in these mixtures contained 2 mole % of (12-DABS)-18 PC, a nonexchangeable quencher of the probe fluorescence (Silvius et al., 1987) The spontaneous exchange of this quencher between lipid vesicles is too slow to affect the results of this study (Silvius et al., 1987). The fluorescence signal recorded from these samples was measured before and after the addition of 1% Triton X-100; the latter fluorescence reading allowed the original fluorescence reading to be corrected, where necessary, for possible small variations in the amount of probe from sample to sample (Nichols and Pagano, 1982).

To estimate the fraction of the total lipid that was exposed to the external aqueous medium in preparations of large uni-lamellar PC/PG and PC/PG/cholesterol vesicles, similar vesicles were prepared

containing 5 or 10 mole% PE. The percentage of total PE exposed at the outer surfaces of these vesicles was determined by the TNBS labeling assay of Nordlund et al. (1981). As PE has been shown to adopt a random transbilayer distribution in lipid vesicles with large radii of curvature (Nordlund et al., 1981), this value provides an estimate (typically with a standard error of less than 10% of the estimated value) of the fraction of the total vesicle lipid that is exposed at the external surface.

Phospholipid and sphingomyelin concentrations were determined by the method of Lowry and Tinsley (1974), with the modification that the sample digestion time was extended to 4 hr. Concentrations of glycosphingolipid probes were determined by measurements of probe fluorescence in methanolic solution, using the corresponding labeled sphingomyelin as a standard.

2.4 Results:

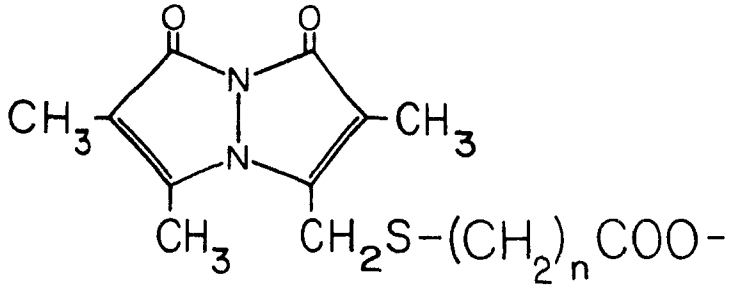
The structures of the fluorescent-labeled fatty acids used in this study are shown in Figure 3. In phosphatidylcholine vesicles, the bimane-, DMCA- and DPHP-labeled lipid species show excitation (emission) maxima at 390 nm (468 nm), 400 nm (460 nm) and 364 nm (435 nm) respectively.

In Figure 4 are shown time courses of fluorescence observed when large unilamellar donor vesicles (80:20 egg PC/egg PG), symmetrically or asymmetrically labeled with (8-bimane)-PC as described in Materials and Methods, are mixed with a large (twentyfold) excess of acceptor vesicles prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18 PC. Upon addition of the acceptor vesicles, the fluorescence signal recorded from either sample rapidly falls to a new plateau value, which remains stable when the vesicles are further incubated for times up to at least a few hours. A much greater fraction of the probe is readily exchangeable (>97% vs. 55% in the example shown in Figure 4) when asymmetrically rather than symmetrically labeled donor vesicles are used. A still smaller fraction of (8-bimane)-PC (<20%) was readily exchangeable from (vortexed) multilamellar vesicles which were symmetrically labeled with the probe (not shown). It thus appears that only probe molecules that are present at the outer surfaces of the donor vesicles can exchange between vesicles on the time scale of these experiments, and that virtually all of the probe molecules in asymmetrically labeled vesicles become incorporated into the vesicles' outer surfaces (Pagano et al., 1981). Similar results were obtained using all of the other exchangeable fluorescent probes examined in this study. Asymmetrically labeled vesicles were therefore used in all further experiments described in this paper in order to maximize the fraction of the probe that was readily exchangeable.

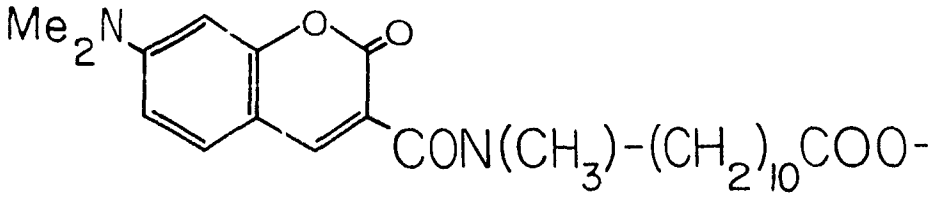
In Figure 5 are shown the time courses of the fluorescence changes observed when a fixed amount of large unilamellar 80:20:2 egg PC/egg PG/(12-DABS)-18 PC vesicles, asymmetrically labeled with (8-bimane)-PE to a final level of 1 mol%, was incubated with varying amounts of acceptor vesicles prepared from 80:20 egg PC/DOPG. It can

Figure 3.

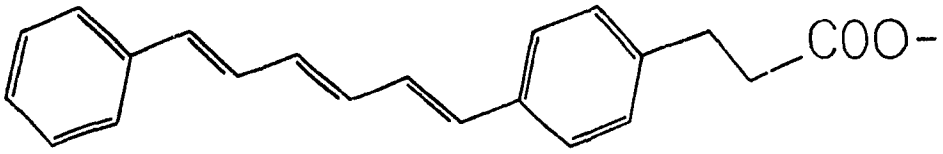
This figure shows the structures of the fluorescent fatty acyl chains attached to the exchangeable lipid probes used in this study. For bimane- and DMCA-labeled phospholipid probes, the labeled acyl chain was attached to the 2-position of a phospholipid carrying a palmitoyl chain at the 1-position. For DPHP-labeled probes, the labeled chain was attached to the 2-position of a phospholipid carrying a decanoyl group at the 1-position.



BIMANE



DMCA



DPHP

Figure 4.

Time courses of fluorescence changes observed when large unilamellar 80:20 egg PC/egg PG vesicles (2.5 μM), labeled symmetrically or asymmetrically with 2 mole% (8-bromano)-PC as described in Materials and Methods, were mixed at time zero with bath-sonicated 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles (100 μM). The dashed horizontal line represents the fluorescence measured for an equal amount of (8-bromano)-PC incorporated into 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles.

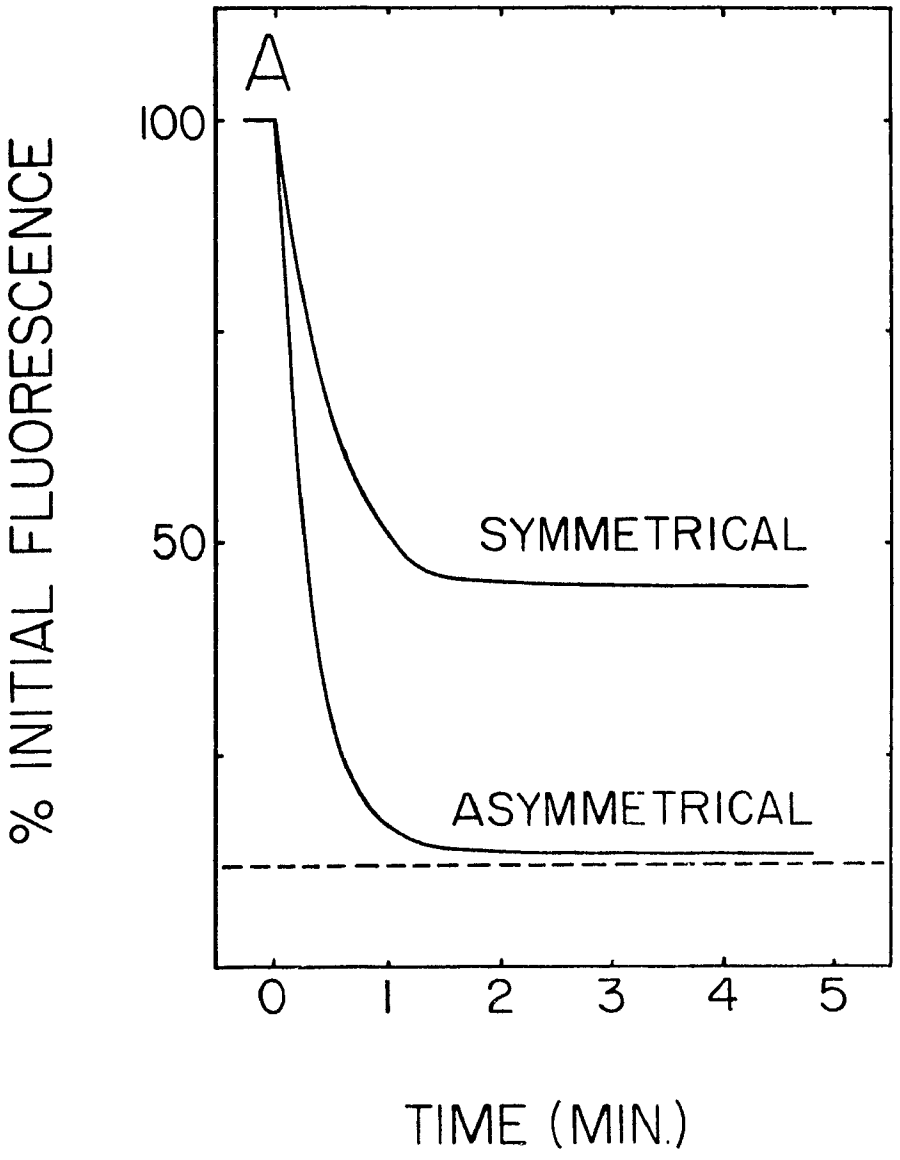
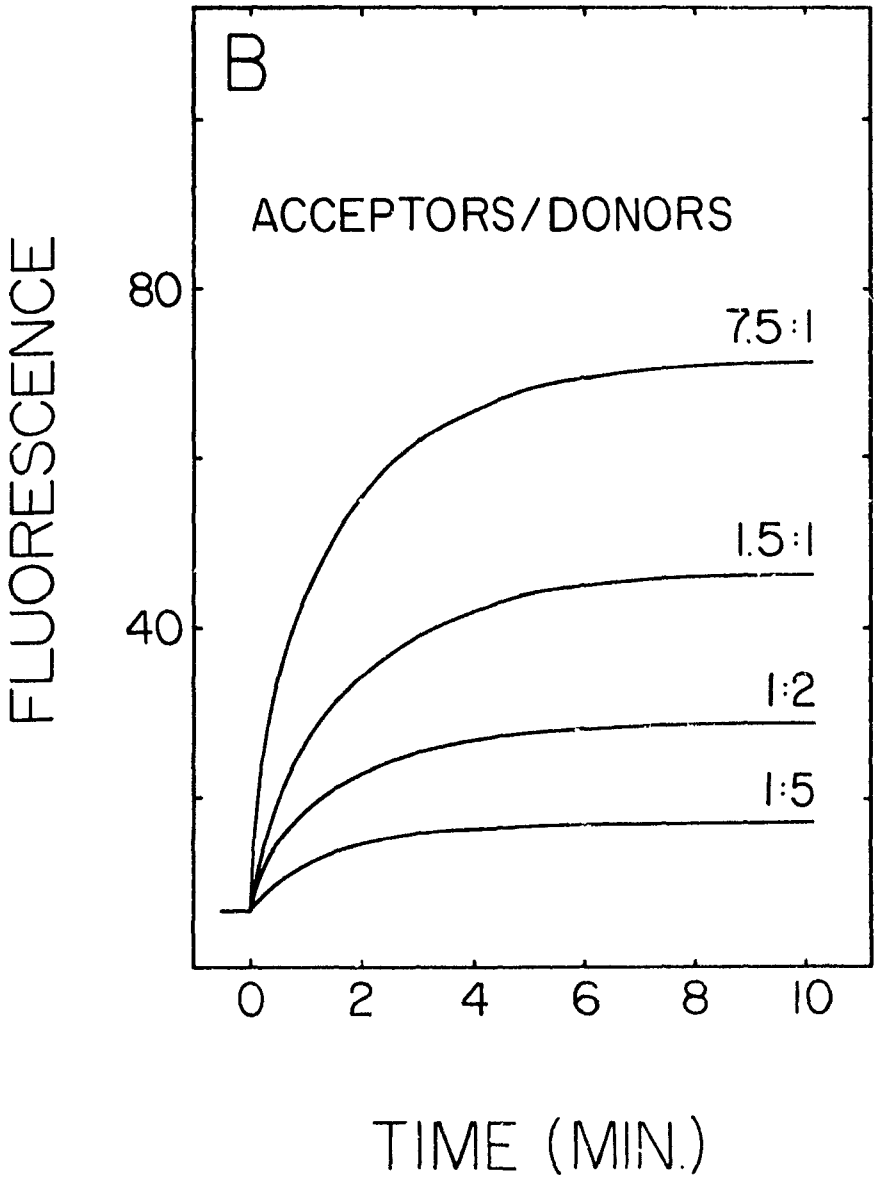

A
100
50
% INITIAL FLUORESCENCE
SYMMETRICAL
ASYMMETRICAL
0
1
2
3
4
5
TIME (MIN.)

Figure 5.

Time courses of fluorescence changes observed when 80:20:2 egg PC/egg PG/(12-DABS)-PC donor LUV (5μM), asymmetrically labeled with 1 mole% (8-bimane)-PE, were mixed at time zero with 80:20 eggPE/DOPS acceptor LUV at the indicated ratios of donor to acceptor vesicles. All traces have been plotted to a common fluorescence scale


B
ACCEPTORS/DONORS
7.5:1
1.5:1
1:2
1:5
80
40
FLUORESCENCE
0
2
4
6
8
10
TIME (MIN.)

be seen that while the amplitudes of the fluorescence changes observed depend strongly on the amount of acceptor vesicles added, the half-time for equilibration of the probe between the vesicle populations is constant over a wide range of acceptor vesicle concentrations. This result suggests that the mechanism of probe transfer does not involve collisions between vesicles (Roseman et al, 1980, Nichols and Pagano, 1981, 1982). Under these conditions, the plateau fluorescence values obtained for a given acceptor/donor vesicle ratio can be plotted in order to obtain an effective partition coefficient ($S * K_p$). These calculations are further described in the appendix to this chapter.

2.4.1 Partitioning of Lipid Probes between Different Vesicle Populations

2.4.1.1 Effects of Phospholipid Composition:

Many of the physical properties of polar lipids have been suggested to depend strongly on the abilities of the lipid headgroups to participate in hydrogen-bonding interactions with the headgroups of neighboring lipids in the membrane bilayer (for reviews, see Boggs, 1980, 1987). To evaluate whether the hydrogen-bonding ability of a lipid headgroup can affect its tendency to associate with 'hydrogen-bonding' lipids in preference to 'non-hydrogen-bonding' lipids, I measured the partitioning of various phospho- and sphingolipid probes between large unilamellar vesicles which were composed either of egg PE and DOPS or of egg PC and egg PG or O-methyl PA. Amino phospholipids such as PE and PS can serve both as hydrogen-bond donors and acceptors and are considered to be good hydrogen-bonding species. By contrast, lipids such as PC can serve only as hydrogen-bond acceptors and are considered to be poor hydrogen-bonding species (Boggs, 1980, 1987; Hauser et al., 1981).

Table 3 summarizes the results of a series of measurements of the partitioning of various lipid probes between 80:20:2 (molar proportions) egg PC/egg PG/(12-DABS)-18 PC LUV and 80:20 egg PE/DOPS

Table 3.

[a]Large unilamellar vesicles, prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18 PC or from 80:20 egg PE/DOPS, were asymmetrically labeled with the indicated lipid probes at 1 mole%. The fluorescence of labeled donor vesicles (3 or 5 uM) was measured after incubation (30 min. for [bimane-octanoyl]-labeled species, 2 hr. for other probes) in the presence of varying concentrations of acceptor vesicles (typically 0.5 to 60 uM; egg PE/DOPS vesicles were used as acceptors with egg PC/egg PG/(12-DABS)-PC donor vesicles, and vice versa). The fluorescence measurements obtained were used to determine effective partition coefficients (K_p'S) as described in the Appendix. For presentation in this table, the effective partition coefficient measured for a given probe in each experiment has been divided by the effective partition coefficient measured for the corresponding PC probe in the same experiment.

[b]80:20:2 egg PC/O-methyl PA/(12-DABS)-18 PC vesicles were used in place of egg PC/egg PG/(12-DABS)-18 PC in these experiments.

[c]Values in this table and in Table 4 are presented as the mean ± S.E.M. from at least three separate determinations.

Table 3.

Partitioning of Phospho- and Sphingolipid Probes Between PC/PG and PE/PS Large Unilamellar Vesicles[a]

Probe	Labeled acyl chain	K_p(Probe)/K_p(PC Probe)
PE	Bimane-octanoyl	0.68 + 0.06
PE[b]	Bimane-octanoyl	0.59 + 0.08
PE	DMCA-undecanoyl	0.68 + 0.09
PE	DPHP	0.83 + 0.03
PG	Bimane-octanoyl	0.90 + 0.07
PG	DMCA-undecanoyl	0.79 + 0.06
PS	Bimane-octanoyl	1.17 + 0.05
PS	DMCA-undecanoyl	1.42 + 0.19
PA	Bimane-octanoyl	1.76 + 0.07
PA[b]	Bimane-octanoyl	1.82 + 0.16
PA	DMCA-undecanoyl	1.73 + 0.24
PA	DPHP	1.40 + 0.15
Sphingomyelin	Bimane-undecanoyl	1.24 + 0.06
Sphingomyelin	DMCA-undecanoyl	1.04 + 0.05
Cerebroside	Bimane-undecanoyl	0.81 + 0.08
Cerebroside	DMCA-undecanoyl	0.66 + 0.08
Sulfatide	DMCA-undecanoyl	0.94 + 0.05

LUV. In this table, the relative affinity of each probe for PE/PS vesicles *vs*. PC/PG vesicles is expressed as the ratio of the partition coefficient K_p for that probe to the partition coefficient measured in the same experiment for the corresponding PC probe. Data were collected both by labeling PC/PG/(DABS-PC) vesicles with the exchangeable probe and titrating with PE/PS vesicles, and in the reciprocal experiment in which PE/PS vesicles were initially labeled with the probe and were titrated with PC/PG/(DABS-PC) vesicles. The results obtained using either method generally agreed very well, and the data shown in Table 3 summarize results obtained from both types of measurements.

Several important points are evident from the data presented in Table 3. First, the partition coefficients for the various lipid probes tested in this system all lie within a factor of two of the values determined for the corresponding PC probes. (For reference, the effective partition coefficients [$S K_p$] measured for the PC probes in these experiments averaged 0.66 for (8-bimane)-PC, 0.49 for (11-DMCA)-PC and 0.85 for DPHP-PC). Second, the effects of a probe's headgroup structure on its relative affinity for PE/PS *vs* PC/PG vesicles are quite consistent regardless of whether the probe carries a bimane-, a coumarin- or DPH-labeled acyl chain. This quantitative consistency of the partitioning results obtained with three different families of probes is noteworthy, since the structures of the fluorescent groups and the flexibilities of the labeled acyl chains differ markedly for the three probe types. Third, the selectivity of a given probe for PE/PS vesicles *vs*. PC/PG vesicles cannot be systematically correlated with the hydrogen-bonding ability of the probe headgroup. For example, PE and monoionized PA are both hydrogen-bonding species while PC is not (Boggs, 1980, 1987), yet PE probes show a lower relative affinity, and PA probes a greater relative affinity, for PE/PS vesicles (in competition with PC/PG vesicles) than do PC probes. Essentially identical results were obtained using PC/O-methyl PA vesicles in place of PC/PG vesicles in these experiments.

2.4.1.2 Effects of Cholesterol:

In a final series of experiments, I examined the partitioning of several bimane-labeled phospho- and sphingolipid probes between LUV composed of either 80:20 egg PC/egg PG/(12-DABS)-18 PC or 48:12:40 egg PC/egg PG/cholesterol. The results of these experiments are summarized in Table 4, where the effective partition coefficient measured for each probe has been normalized to that measured for the corresponding PC probe in the same experiment. The relative affinities of various probes for cholesterol-containing vs. cholesterol-free vesicles decrease in the order sphingomyelin > cerebroside > sulfatide ≈ PC > PE. Again, however, the different probes vary only modestly in their relative affinities for cholesterol-containing vs. cholesterol-free vesicles. The partition coefficient measured for the anionic sulfatide probe in these experiments may be slightly elevated, in favour of the cholesterol-containing vesicles, by the fact that the surface charge density is somewhat lower in the cholesterol-containing than in the cholesterol-free vesicles. However, by examining the effect of surface charge on lipid partitioning, one can estimate that this effect will be small (enhancing K_p by no more than 20-25%) under the conditions of these experiments (Gaidam et al., 1989)

The value of the effective partition coefficient ($S.K_p$) measured for the partitioning of (11-bimane)-11 PC between cholesterol-containing and cholesterol-free vesicles in the above experiments averaged 0.191 ± .027 in six independent determinations. The results of TNBS-labeling experiments, carried out using similar vesicles containing 5 mole% egg PE as described in Materials and Methods, indicated that essentially the same fraction of the vesicle lipid was exposed at the outer surfaces of both cholesterol-containing and cholesterol-free vesicles (i.e., that $S \approx 1$). It thus appears that cholesterol-containing vesicles exhibit a substantially (3- to 6-fold) lower affinity for all of the probes studied here than do similar vesicles that do not contain cholesterol.

Table 4.

[a]Large unilamellar vesicles, prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18 PC or 48:12:40 egg PC/egg PG/cholesterol, were asymmetrically labeled with the indicated probes at 1 mole%. The partitioning of the probes between these two types of vesicles was determined as described for the experiments summarized in Table 3. The partition coefficient for a given probe is defined as the ratio of the affinities of the probe for the cholesterol-containing vesicles over the cholesterol-free vesicles. For presentation in this table, the partition coefficient measured for each probe has been divided by that measured for (11-DMCA)-PC in the same experiments.

Table 4.

Partitioning of Lipid Probes Between Large Unilalmellar PC/PG Vesicles and PC/PG/Cholesterol Vesicles[a]

Probe	K_p(Probe)/K_p(PC)
N-(11-DMCA)-sphingomyelin	1.99 + 0.18
N-(11-DMCA)-sulfatide	1.31 + 0.09
N-(11-DMCA)-cerebroside	1.79 + 0.09
(11-DMCA)-PE	0.77 + 0.14

2.5 Discussion:

Previous analyses of phase diagrams for various binary mixtures of lipids with different headgroups (Shimshick and McConnell, 1973; Wu and McConnell, 1975; Mabrey and Sturtevant, 1976; Arnold et al., 1981; Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986, 1987) have suggested that such lipids often show significantly non-ideal mixing even in liquid-crystalline lipid bilayers. These results have important implications for understanding the microscopic organization of multicomponent lipid membranes, including such aspects as the lateral distributions of different lipid species (Von Dreele, 1978; Knoll et al., 1985; Thompson and Tillack, 1985; Somerharju et al., 1985), the energy required to create and to maintain transmembrane lipid asymmetry (Tenchov and Koynova, 1985), and the local formation of nonlamellar structures in mixtures of 'bilayer-forming' and 'non-bilayer-forming' lipids (Vasilenko et al., 1982; Tilcock et al., 1982; Boni and Hui, 1983; Siegel, 1984; Eriksson et al., 1985; Tate and Gruner, 1987). It therefore seems desirable to utilize complementary approaches, such as direct measurements of lipid partitioning between different lipid environments (Lange et al., 1979; Nichols and Pagano, 1982; Wattenberg et al., 1983; Fugler et al., 1985; Rujanavech and Silbert, 1986; Yeagle and Young, 1986) to confirm the conclusions of the above studies and to extend them to more complex systems (e.g., mixtures of lipids with heterogeneous acyl chain compositions or mixtures of lipids and sterols), for which a rigorous determination of the phase diagram may not be feasible.

A somewhat surprising finding of this study is that lipid probes with a variety of different headgroups vary only modestly in their relative affinities for different types of phospholipid bilayers. As already noted, previous studies of the behaviour of binary lipid mixtures have suggested that the mixing of lipids with different polar headgroups can be markedly nonideal even in the liquid-crystalline state. As a case in point, one can estimate from previously reported results (Lee, 1977; Arnold et al., 1981) that the excess enthalpy of mixing of a low mole fraction of dimyristoyl or dipalmitoyl

PE with the corresponding PC in a liquid-crystalline bilayer will be on the order of 1.0 kcal mol^{-1}. If the exchangeable PE and PC probes examined in this study were to show a similar discrimination in their interaction with PE vs. PC in lipid bilayers, one would predict that a PE probe would show roughly a five-fold greater relative affinity for PE-rich vesicles, in competition with PC-rich vesicles, than would the corresponding PC probe. In fact, I have found that exchangeable PE probes show a slightly lesser preference for PE-rich vesicles vs. PC-rich vesicles than do the corresponding PC probes.

The observation that PE probes fail to discriminate in favour of a 'hydrogen-bonding' lipid environment (PE/PS vesicles) over a 'non-hydrogen-bonding' environment (PC/PG or PC/O-methyl PA vesicles) is not inconsistent with the suggestion that the amino group of PE may form hydrogen bonds to neighboring phospholipid molecules in lipid bilayers. The PE amino group can in principle hydrogen-bond to the phosphoryl group of any phospholipid, including species such as PC that lack hydrogen bond-donating groups. The transfer of PE molecules from a PE-rich to a PC-rich environment, either within one bilayer or between distinct lipid vesicles, thus need not decrease the total number of hydrogen bonds between lipid headgroups in the system as a whole. The same is true for the transfer of PC molecules from a PC-rich to a PE-rich environment. There thus need be no inherent energetic 'cost' at least in terms of a loss of hydrogen-bonding interactions, that accompanies the intermixing of PE and PC molecules in lipid bilayers.

The values obtained for the partitioning of various lipid probes between PC/PG vesicles and PC/PG/cholesterol vesicles (Table 4) indicate that the structure of the probe headgroup has a significant, if modest influence on the relative affinity of the probe for cholesterol-containing vs. cholesterol-free bilayers. The relative affinities of different lipid probes for cholesterol-rich bilayers decrease in the order sphingomyelin > PC > PE, which coincides with the order of affinities of these three lipids for cholesterol as deduced from previous calorimetric studies (van Dijck et al. 1976, 1979, Demel et al., 1977) and some cholesterol-partitioning measure-

ments (Wattenberg et al., 1983; Fugler et al., 1985; Yeagle and Young, 1986; for a contrasting report, see Lange et al., 1979). In this system also, however, it is noteworthy that lipid probes with different headgroups differ only modestly in their discrimination between a cholesterol-free and a cholesterol-containing lipid bilayer environment.

In any study involving the use of fluorescent-labeled lipids to elucidate the behaviour of unlabeled lipids, it is important to ensure that the results obtained with the fluorescent lipid adequately reflect the behaviour of the corresponding unlabeled species. I believe that this condition is fulfilled for the thermodynamic experiments described here for the following reasons. First, the major conclusions in this study are based on comparisons of the behaviour of probes with different headgroups but the same fluorescent-labeled acyl chains. Such comparisons should serve to factor out the possible effects of the fluorescent group per se on the partitioning of lipid probes between different bilayer environments. In support of this suggestion, one should note that such comparisons reveal consistent effects of the probe headgroup structure on the partitioning of three different families of fluorescent probes, with quite different structures of their labeled acyl chains, between PC/PG and PE/PS vesicles. Second, kinetic studies (summarized in Gardner et al., 1989) demonstrate that the headgroup structure of a lipid probe significantly affects its rate of desorption from a lipid bilayer, in a manner that is observed consistently for the various families of probes examined in this study as well as for a previously studied series of pyrene-labeled phospholipids (Marcus et al., 1982). It thus appears that the presence of fluorescent reporter group on the lipid probes examined here does not eliminate the possibility of headgroup-specific interactions between the probes and the surrounding lipid molecules. For these reasons, I feel that the result obtained in this study provide a valid basis to evaluate possible selectivities in the interactions between lipids with different polar headgroups in liquid-crystalline lipid bilayers.

These findings suggest that the intermixing of lipids with different headgroups in liquid-crystalline bilayer membranes may be more nearly ideal than would be inferred from previous analyses of phase diagrams for binary lipid mixtures (see, e.g., Lee, 1977; Arnold et al., 1981; Maggio et al., 1985). However, the apparent discrepancies between some of the present conclusions and those derived from studies of the type just cited may rest on certain key differences in the experimental systems and analyses employed in each case. First, the present experiments have used samples composed of (noninteracting) unilamellar lipid vesicles, while phase diagrams for multicomponent lipid mixtures are typically obtained using samples in which different bilayers can readily interact at close range (e.g., dispersions of multilamellar vesicles). It is possible that the mixing of different phospholipids may be appreciably different in large unilamellar vs multilamellar structures. Second, quantitative analyses of the phase diagrams for binary lipid mixtures (e.g., using regular solution theory) require simultaneous evaluation of the mixing of the lipid species in the solid (gel) and liquid-crystalline phases. Even simple lipid mixtures often exhibit multiple coexisting solid phases (Luna and McConnell, 1977, 1978; Stewart et al., 1979; Grabitz et al., 1985; Silvius, 1986), a fact that is not usually allowed for in simple regular-solution analyses. It may be difficult to analyze the phase diagrams for such systems rigorously to extract information about the mixing of lipids in the liquid-crystalline state. Finally, most of the binary lipid systems for which phase diagrams are presently available consist of lipids with saturated and trans-unsaturated acyl chains. It is possible that the effects of headgroup structure on the intermixing of other types of lipids (e.g., 1-saturated-2-cis-unsaturated species, or species with heterogeneous acyl chain compositions) may be rather different from those observed in the systems just noted.

In conclusion, the results presented in this study indicate that lipids with different headgroups may intermix in liquid-crystalline bilayers in a manner that is more nearly ideal than some previous studies have suggested. One major advantage of the partitioning

technique described here (and one possible source for the discrepancies between the present conclusions and those derived from previous thermodynamic studies of binary lipid mixtures) is the fact that the partitioning approach can be applied even to systems that contain a number of different molecular components. This approach may thus be useful not only to investigate lipid-lipid interactions in simple vesicle systems but also to test for possible selectivity in lipid-lipid and lipid-protein interactions in more complex systems, including biological membranes.

2.6 Appendix: Equilibrium Partitioning of Labeled Probes Between Lipid Vesicles:

The simplest model of partitioning of an exchangeable lipid probe I* between two populations of lipid vesicles can be described by the equation

$$[1] \qquad \frac{X_{I^*}(\text{Acceptor})}{X_{I^*}(\text{Donor})} = K_p$$

where X_{I^*}(Acceptor) and X_{I^*}(Donor) represent the mole fractions of the probe in the external surfaces of the acceptor and donor vesicles, respectively, and K_p is a partition coefficient that is determined by the free energy of transfer of the probe from the donor to the acceptor vesicles. Applying the above definition and simple considerations of mass balance to describe the distribution of an exchangeable fluorescent probe between two different types of lipid vesicles, a straightforward derivation gives us the more directly useful equation

$$[2] \qquad F_{norm} = F_D + (F_{max}) * \frac{S * K_p * [\text{Acceptors}]}{[\text{Donors}] + S.K_p [\text{Acceptors}]}$$

where

$$[3] \qquad F_{max} = (F_{Ac} - F_D).(f_{exch})$$

In the above equations, the normalized fluorescence F_{norm} is the fluorescence measured for a given probe-containing sample, divided by the fluorescence measured after the addition of excess Triton X-100 (which provides a direct measure of the amount of probe present). F_D is the normalized fluorescence of the probe when present in the donor vesicles, F_{Ac} is the normalized fluorescence of the probe when present in the acceptor vesicles, and f_{exch} is the fraction of the total probe that is readily available for exchange. Finally, S represents the fraction of total lipids in the acceptor vesicles that is exposed to the external medium, divided by the corresponding value for the donor vesicles.

In my experiments, values for the parameters $(S.K_p)$ and F_{max} were estimated by applying equation [2] to sets of fluorescence data obtained by incubating a small and fixed amount of a fluorescent probe with a fixed concentration of donor vesicles and variable concentrations of acceptor vesicles. Plots of I_{norm} vs the acceptor vesicle concentration were fit directly to equation [2], using a nonlinear least-squares fitting routine employing a Marquardt algorithm, to yield F_D, F_{max} and $(S.K_p)$ as estimated parameters. In a typical experiment, 20-22 data points were collected per curve, and the mean errors of estimation for F_{max} and $(S.K_p)$ were of the order of 1-2% and 3-5% of the estimated values, respectively. Since F_D and F_{AC} can be determined directly, it is possible to estimate f_{exch} as well from the estimated value for F_{max}.

The analysis just described can estimate an effective partition coefficient $(S.K_p)$ but not K_p itself. This limitation can be overcome in two ways. First, one can estimate the value of S by an independent experiment; in this study, this was achieved by applying the INBS labeling assay of Nordlund et al. (1981) to estimate the fractions of lipids exposed at the outer surfaces of various types of vesicles that contained low mole fractions of PE (see Materials and Methods). Second, by comparing the $(S.K_p)$ values measured for the partitioning of two different probes between two populations of vesicles, one can calculate the ratio of the K_p values for the partitioning of the two probes between different lipid environments.

CHAPTER THREE:

Intermixing of Dipalmitoyl Phosphatidylcholine with Phospho- and Sphingolipids Bearing Highly Asymmetric Hydrocarbon Chains

3.1 Abstract[1]:

High-sensitivity differential scanning calorimetry has been employed to investigate the mixing of dipalmitoyl phosphatidylcholine (DPPC) with N-lignoceroyl galactocerebroside, N-lignoceroyl sulfogalactocerebroside and 1-lauroyl-2-lignoceroyl phosphatidylcholine. These three lignoceroyl species, whose two hydrocarbon chains are quite discrepant in length, are completely miscible with DPPC in the liquid-crystalline state. Mixtures of all three lignoceroyl lipids with DPPC show phase separation in the gel state, which is observed over a limited range of compositions (from <10 mole% to just over 40 mole% sulfatide) in the case of N-lignoceroyl sulfatide and over a wide range of compositions in the cases of N-lignoceroyl cerebroside (<10 mole% to >90 mole% cerebroside) and 1-lauroyl-2-lignoceroyl PC (roughly 10 mole% to 90 mole% lauroyl/lignoceroyl PC). The extensive solid-solid phase separation observed in mixtures of DPPC and 1-lauroyl-2-lignoceroyl PC, which show eutectic behavior, is somewhat unexpected given the similar transition temperatures of the two components but appears to reflect the ability of the lignoceroyl species to form an interdigitated gel phase. However, the present study finds no evidence that the N-lignoceroyl sphingolipids are markedly more prone to segregate laterally in PC-rich bilayers than are previously studied sphingolipid species with shorter N-acyl chains. It is suggested on the basis of these results that the primary biological importance of the very long N-acyl chains found in many sphingolipids may lie in some function other than the promotion of lateral segregation of sphingolipid-enriched domains in biological membranes.

---

[1]The material in this chapter has been published as Curatolo, W. and Silvius, J. F. (1989) Biochim. Biophys. Acta 986, 319-327.

3.2 Introduction:

It is a striking fact that some of the lipids found in mammalian cell membranes exhibit structures that depart strongly from the 'consensus' pattern exemplified by a diacylglycerophospholipid with acyl chains of roughly equal length. One noteworthy example of this phenomenon is the occurrence in mammalian cell plasma membranes of sphingolipids with N-acyl chains whose length greatly exceeds that of the hydrocarbon portion of the sphingosine backbone (O'Brien and Rouser, 1964; Rouser et al., 1968). A variety of 'asymmetric' phospho- and sphingolipids, whose two hydrocarbon chains differ markedly in length, have been reported to form interdigitated phases, in which the longer of the two hydrocarbon chains extends well beyond the bilayer midplane (Mason et al., 1981; Huang et al., 1983; McIntosh et al., 1984; Hui et al 1984; Levin, et al., 1985; Boggs and Mason, 1984; Huang and Mason, 1986, Maulik et al., 1986, Reed and Shipley, 1987, Mattai et al., 1987, Xu and Huang, 1987; Boggs et al., 1988) To date, relatively little is known about the effects of this dramatic asymmetry in chain length on the abilities of such lipids to intermix with other lipids whose hydrocarbon chains are more nearly comparable in length. Lateral segregation of interdigitated and noninterdigitated phases has been reported in a few binary lipid systems, including one in which a liquid-liquid as well as solid-solid phase separation has been reported (Lohner et al., 1987; Kim et al 1988, Mason 1988)

In the light of the above considerations, it is of interest to examine the miscibility of 'asymmetric' lipids, such as the naturally occurring N-lignoceroyl sphingolipids, with 'symmetric' phospholipids (i.e., phospholipids whose two acyl chains are of similar length) of the type that comprise the major fraction of natural membrane phospholipids The potential of the former species to form interdigitated phases in isolation (Levin et al., 1985, Maulik et al., 1986, Reed and Shipley, 1987; Boggs et al., 1988) could in principle favor their lateral segregation from symmetrical phospholipids in mixed lipid bilayers or even in biological membranes. To test this

possibility, I have examined the mixing of a symmetrical phospholipid, dipalmitoyl phosphatidylcholine (DPPC), with the highly asymmetric lipids N-lignoceroyl galactocerebroside (N-24:0 cerebroside), N-lignoceroyl 3'-sulfogalactocerebroside (N-24:0 sulfatide), and 1-lauroyl-2-lignoceroyl phosphatidylcholine (12:0/24:0 PC). A particularly interesting phase diagram is obtained for the DPPC/1-Lauroyl-2-lignoceroyl PC system, which exhibits clear eutectic behavior. However, there is no evidence suggesting that the lignoceroyl species examined here are significantly more prone to segregate laterally in a liquid-crystalline phospholipid bilayer than are other lipids with the same headgroups but more nearly symmetrical hydrocarbon chains.

3.3 Materials and Methods:

3.3.1 Materials

Galactocerebroside and 3'-sulfogalactocerebroside (sulfatide) were isolated from an acetone powder of bovine brain by Folch extraction, then purified by silicic acid and ion-exchange chromatography, using methods described previously (Radin, 1976, Momoi et al., 1976). N-Lignoceroyl galactocerebroside was synthesized from brain cerebroside by alkaline deacylation (Radin, 1974) followed by reacylation with lignoceric acid in the presence of triphenylphosphine and 4,4'-bispyridyldisulfide (Kishimoto, 1975). N-lignoceroyl sulfatide was synthesized starting from bovine brain sulfatide by the procedure of Koshy and Boggs (1982, 1983). 1-Lauroyl-2-lignoceroyl phosphatidylcholine was synthesized, starting from dilauroyl phosphatidylcholine, by the procedure of Mason et al. (Mason et al., 1981). These lipids were purified by silicic acid column chromatography followed by preparative thin-layer chromatography and a final precipitation from chloroform with cold acetone. Dilauroyl and dipalmitoyl phosphatidylcholine (99+%) were obtained from Sigma (St. Louis, Mo.).

3.3.2 Methods

Lipid samples were lyophilized from cyclohexane and dispersed in 156 mM NaCl, 10 HEPES, 1 mM EDTA, pH 7.4 by vortexing above the phase transition of the higher-melting component. Samples containing N-lignoceroyl cerebroside, N-lignoceroyl sulfatide or 1-lauroyl-2-lignoceroyl PC were very briefly heated to 90°C, to 65°C or to 60°C, respectively, then cooled rapidly to 55°C and cooled from the latter temperature to 4°C at a rate less than 0.3 degrees per minute. Except where otherwise explicitly indicated, samples were then incubated at 4°C for 45 to 60 days before calorimetric analysis.

High-sensitivity differential scanning calorimetric analysis of lipid samples was carried out with a Microcal MC-1 scanning

calorimeter, using a scan rate of 12°C/hr. Equilibrated samples (5 umoles total lipid in 0.7 ml buffer) were loaded into the calorimeter at temperatures below 5°C and were scanned to a temperature at least 10° above the transition temperature of the higher-melting lipid component. In some experiments, samples were then recooled to 5°C at a rate of roughly 1°C/min. and rescanned within 2 hr. Concentrations of lipid stock solutions were determined by phosphorus analysis (Lowry and Tinsley, 1974) for phosphatidylcholines and by weighing thoroughly dried samples for N-lignoceroyl galactocerebroside and N-lignoceroyl sulfogalactocerebroside. Phase transition boundaries were estimated from thermograms recorded for mixed-lipid samples using the procedure of Mabrey and Sturtevant (1976).

3.4 Results:

3.4.1 DPPC / N-lignoceroyl Cerebroside Mixtures

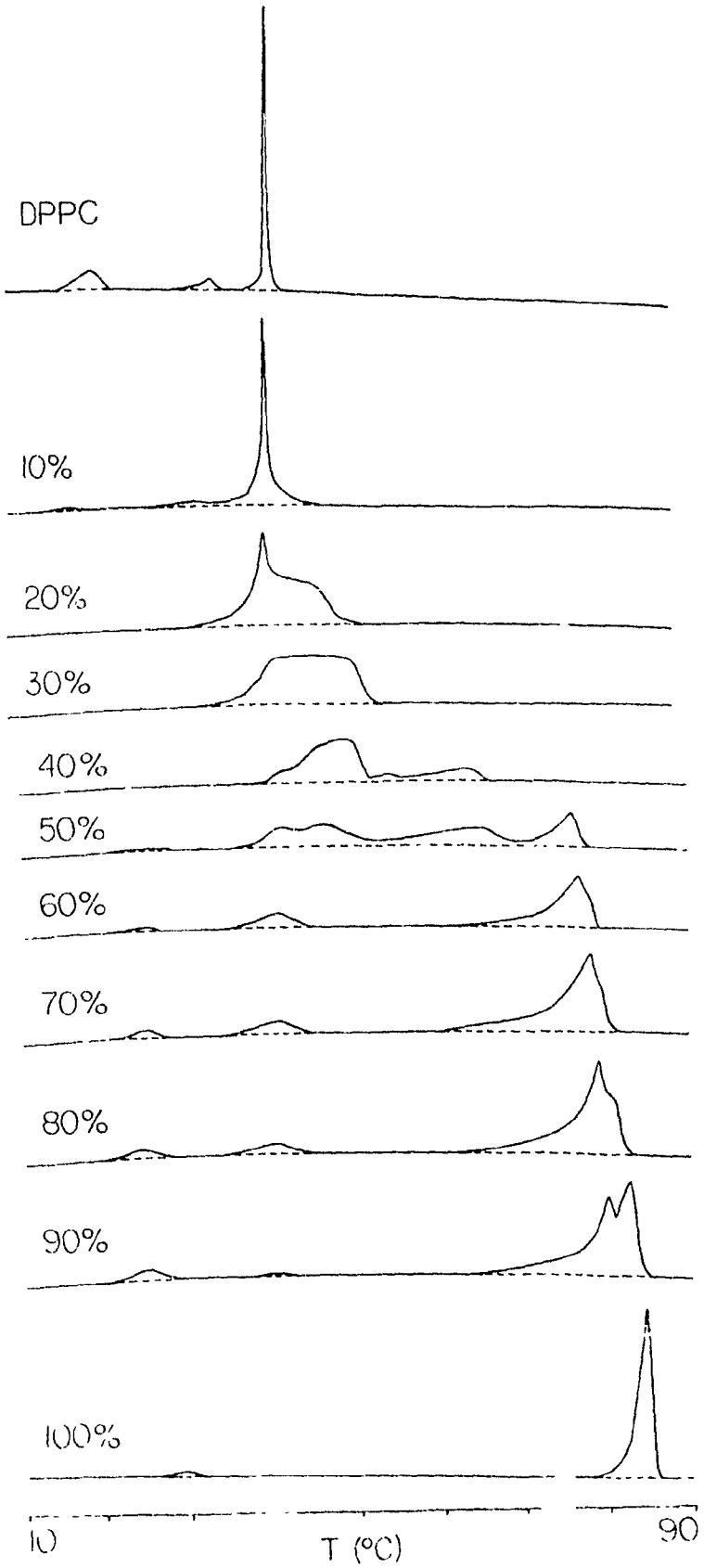
In Figure 6 are shown a series of calorimetric traces obtained for extensively preincubated samples combining DPPC with N-24:0 cerebroside in varying proportions. The preparation of DPPC used in these experiments, after incubation for 45 days at 4°C, exhibits sub-, pre- and main transitions at 20.6°C, 35.0°C and 42.0°C, respectively. Pure N-24:0 cerebroside preincubated under the same conditions shows a small endotherm at 29.4°C and a large endothermic transition at 85.2°C. Samples of N-24:0 cerebroside that were incubated for shorter periods of time (up to 20 days) also showed a second high-temperature transition, centered at 82.9°C (not shown), which is probably the same as the 82°C transition observed by Reed and Shipley (1987) in samples of this lipid that were incubated for times on the order of minutes to hours. The amplitude of the 82.9°C transition gradually decreased, while that of the 85.2°C transition increased, as samples were incubated for increasing times at 4°C. The lower-temperature transition of N-24:0 cerebroside was not observed in samples that were incubated for short times (hours or a few days) prior to calorimetry.

As increasing amounts of N-24:0 cerebroside are incorporated into DPPC bilayers, the main transition of the latter species gradually broadens, with the upper boundary shifting to progressively higher temperatures (Figure 6). However, the lower boundary of the main transition envelope remains near 41.6°C for mixtures containing as much as 90 mole% N-24:0 cerebroside. The pre- and subtransition endotherms observed for DPPC are broadened and shifted to lower temperatures for samples containing 10 mole% cerebroside and cannot be clearly resolved in samples containing 20 mole% or more N-24:0 cerebroside. However, an apparently different low-temperature transition is seen at 25.0°C in samples containing 50-90 mole% cerebroside and at 29.4°C in samples containing 100 mole% cerebroside.

Figure 6.

Heating thermograms recorded for samples combining DPPC with the indicated molar percentages of lignoceroyl cerebroside. After initial dispersal as described in Materials and Methods, samples were incubated for 45 days at 4°C before calorimetric analysis. Other details of the calorimetric analysis were as described in Materials and Methods.

DPPC /24-GAL CER



In Figure 7 is shown the phase diagram derived from the data shown in Figure 6 for the DPPC - N-24:0 cerebroside system. A horizontal line of three-phase coexistence at 41.6°C extends from <10 mole% to over 90 mole% DPPC. A second line of three-phase coexistence is clearly present at 25.0°C, extending from <50 mole% to >90 mole% N-24:0 cerebroside. To account for the observed behavior of the pretransition as N-24:0 cerebroside is introduced into DPPC, and noting that DPPC and N-24:0 cerebroside are almost completely phase-separated at temperatures just below the main phase transition, it is suggested that a third line of three-phase coexistence at a temperature somewhere between 33°C and 25°C (plotted tentatively at 33°C on the phase diagram) exists. This putative feature of the phase diagram may not be clearly resolvable in the thermograms of samples containing 20 mole% or more N-24:0 cerebroside if the relevant endotherm is relatively broad, since the enthalpy involved is small.

The position of the liquidus curve in the phase diagram in Figure 7 indicates that DPPC and 24:0-cerebroside are completely miscible, albeit in a highly nonideal manner, in the liquid-crystalline state. The overall behavior of the liquidus curve is quite similar to that observed by Ruocco *et al.* (1983) in the phase diagram for the DPPC/N-palmitoyl galactocerebroside system.

3.4.2 DPPC / 12:0/24:0 PC Mixtures

In Figure 8 are shown the thermograms recorded for a series of mixtures of DPPC and 12:0/24:0-PC that were incubated for 45 days at 4°C before calorimetric analysis. The effective chain length difference for 12:0/24:0 PC is similar to (albeit slightly greater than) that estimated for N-lignoceroyl sphingolipids (Pascher and Sundell, 1977) and is of the magnitude shown by Xu and Huang (1987) to favor the formation of mixed interdigitated phases in the gel state. Pure 12:0/24:0-PC after lengthy preincubation shows a major phase transition at 49.5°C and a smaller phase transition at 26.0°C. The enthalpies of these transitions are 1.4 ± 0.2 and 13.8 ± 0.4 kcal mole^{-1}, respectively. The larger, but not the smaller transition is also

Figure 7.

Phase diagram deduced from calorimetric data for binary mixtures of DPPC with N-lignoceroyl galactocerebroside (24:0-cerebroside). Phases are designated as follows: L_α, liquid-crystalline; P_β', 'ripple' phase formed by DPPC between the pre- and main transitions; L_β', gel phase formed by DPPC between the sub- and pre-transitions; L_c, subgel phase formed by DPPC; S_1, S_2, upper- and lower-temperature solid phases formed by the lignoceroyl species. Data points plotted represent the onset and completion temperatures, estimated using the procedure of Mabrey and Sturtevant (26), for endothermic events associated with regions of two-phase coexistence in the phase diagrams, as well as the peak temperature measured for endothermic features associated with lines of three-phase coexistence. Phase boundaries that can be reliably assigned from the calorimetric data are indicated by solid lines in the phase diagrams, while boundaries whose position cannot be assigned precisely, or which must be postulated in order to complete the phase diagrams in accordance with the phase rule, are indicated as dashed lines. For clarity, some regions of the phase diagrams are left unlabeled when the phases present can be clearly inferred from the labeling of the adjacent regions.

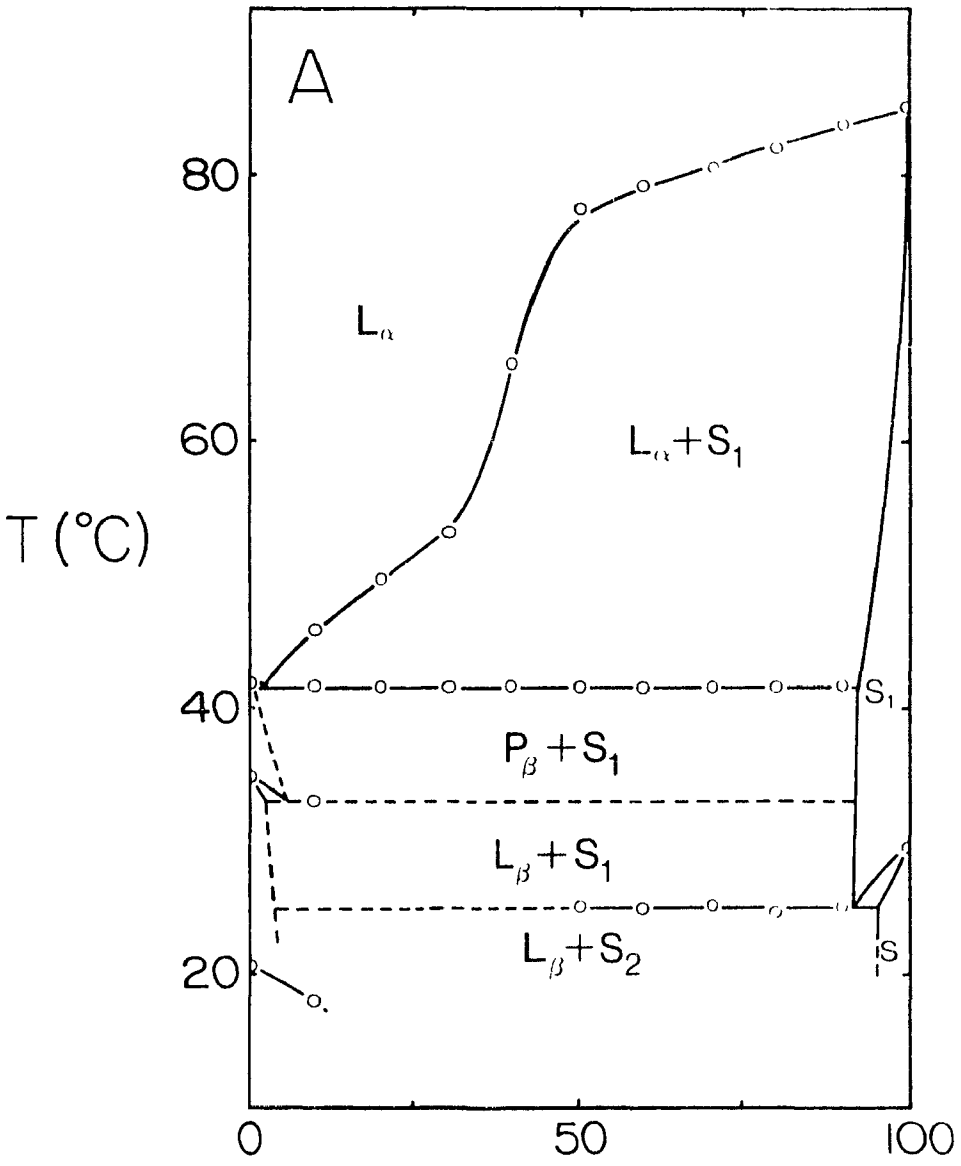
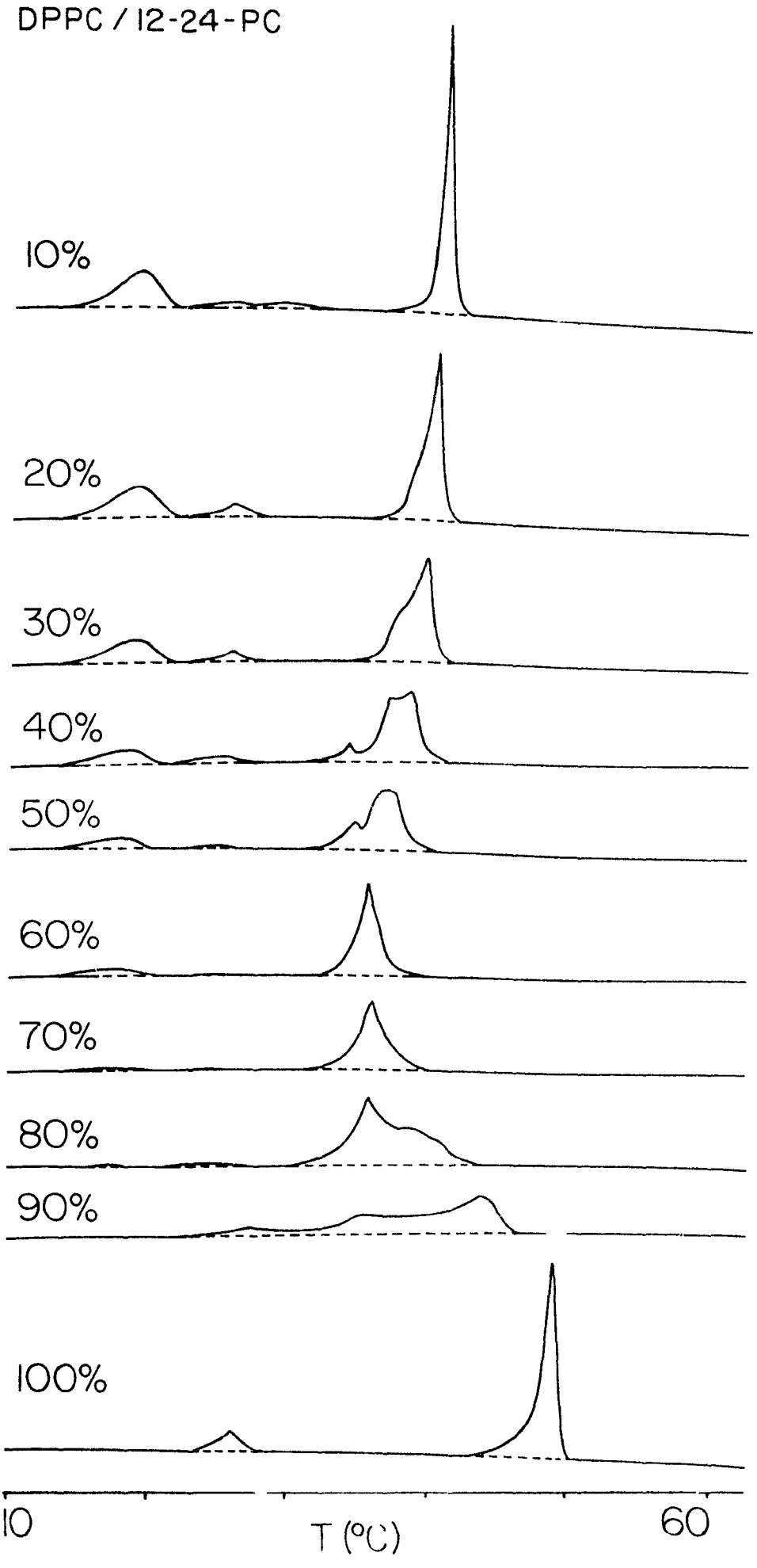

A
80
60
40
20
T (°C)
L_α
$L_\alpha + S_1$
S_1
$P_\beta + S_1$
$L_\beta + S_1$
$L_\beta + S_2$
S
0
50
100
MOLE % CEREBROSIDE

Figure 8.

Heating thermograms recorded for samples of DPPC plus the indicated molar percentages of 12:0/24:0-PC. Samples were after dispersed at 60°C and incubated for 45 days at 4 C prior to calorimetry. Other details of sample preparation and calorimetric analysis are given in Materials and Methods.


DPPC / 12-24-PC
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
10
T (°C)
60

observed in samples that are incubated for shorter times (hours to a few days) at 4°C after heating to 60°C.

As increasing amounts of 12:0/24:0-PC are incorporated into DPPC bilayers, the sub-, pre- and main transitions of the latter species all shift to lower temperatures. The sub- and pretransitions reach new constant temperatures (18.7°C and 25.0°C, respectively) in samples containing ≥10 mole% and ≥20 mole% 12:0/24:0-PC, respectively. The upper boundary of the main transition gradually shifts downward as the mole percentage of 12:0/24:0-PC is increased from 0 to 60 mole%, then gradually rises again from 70 to 100 mole% 12:0/24:0-PC.

In Figure 9 is shown the phase diagram derived from the calorimetric data shown in Figure 8 for the DPPC - 12:0/24:0-PC system. Three lines of three-phase coexistence can be definitely assigned, at 18.7°C, 25.0°C and 35.0°C, respectively. A fourth line of three-phase coexistence must be present between 25°C and 35°C to account for the behavior of the low-temperature transition of 12:0/24:0-PC; this three-phase line is tentatively assigned at 27.5°C. A clear eutectic point is seen at 35.0°C, corresponding to a eutectic composition of roughly 63 mole% 12:0/24:0-PC. Phase separation is seen below 35°C in mixtures containing from roughly 35 mole% up to at least 90 mole% 12:0/24:0-PC. Phase separation at lower temperatures, where the DPPC-rich phase enters the L_β and L_c phases, is even more extensive. The two lipids are fully miscible in the liquid-crystalline phase.

3.4.3 DPPC / N-lignoceroyl Sulfatide Mixtures

In Figure 10 are shown a series of thermograms recorded for extensively preincubated dispersions combining DPPC with N-24:0 sulfatide in various proportions. Pure N-24:0 sulfatide shows a major endothermic transition peaking at 52.8°C, followed by a small transition at 58.5°C. When the sample was cooled rapidly from 65°C after the initial heating run and then immediately rescanned, a similar pattern of endotherms was observed, but the major endotherm in this case was slightly broader and appeared to be the sum of two

Figure 9.

Phase diagram deduced from calorimetric data for binary mixtures of DPPC with 1-lauroyl-2-lignoceroyl phosphatidylcholine (12:0/24:0-PC). Phases are designated as follows: L_α, liquid-crystalline; P_β', 'ripple' phase formed by DPPC between the pre- and main transitions; L_β', gel phase formed by DPPC between the sub- and pre-transitions; L_c, subgel phase formed by DPPC; S_1, S_2, upper- and lower-temperature solid phases formed by the lignoceroyl species. Data points plotted represent the onset and completion temperatures, estimated using the procedure of Mabrey and Sturtevant (26), for endothermic events associated with regions of two-phase coexistence in the phase diagram, as well as the peak temperatures measured for endothermic features associated with lines of three-phase coexistence. Phase boundaries that can be reliably assigned from the calorimetric data are indicated by solid lines in the phase diagrams, while boundaries whose position cannot be assigned precisely, or which must be postulated in order to complete the phase diagrams in accordance with the phase rule, are indicated as dashed lines. For clarity, some regions of the phase diagrams are left unlabeled when the phases present can be clearly inferred from the labeling of the adjacent regions.

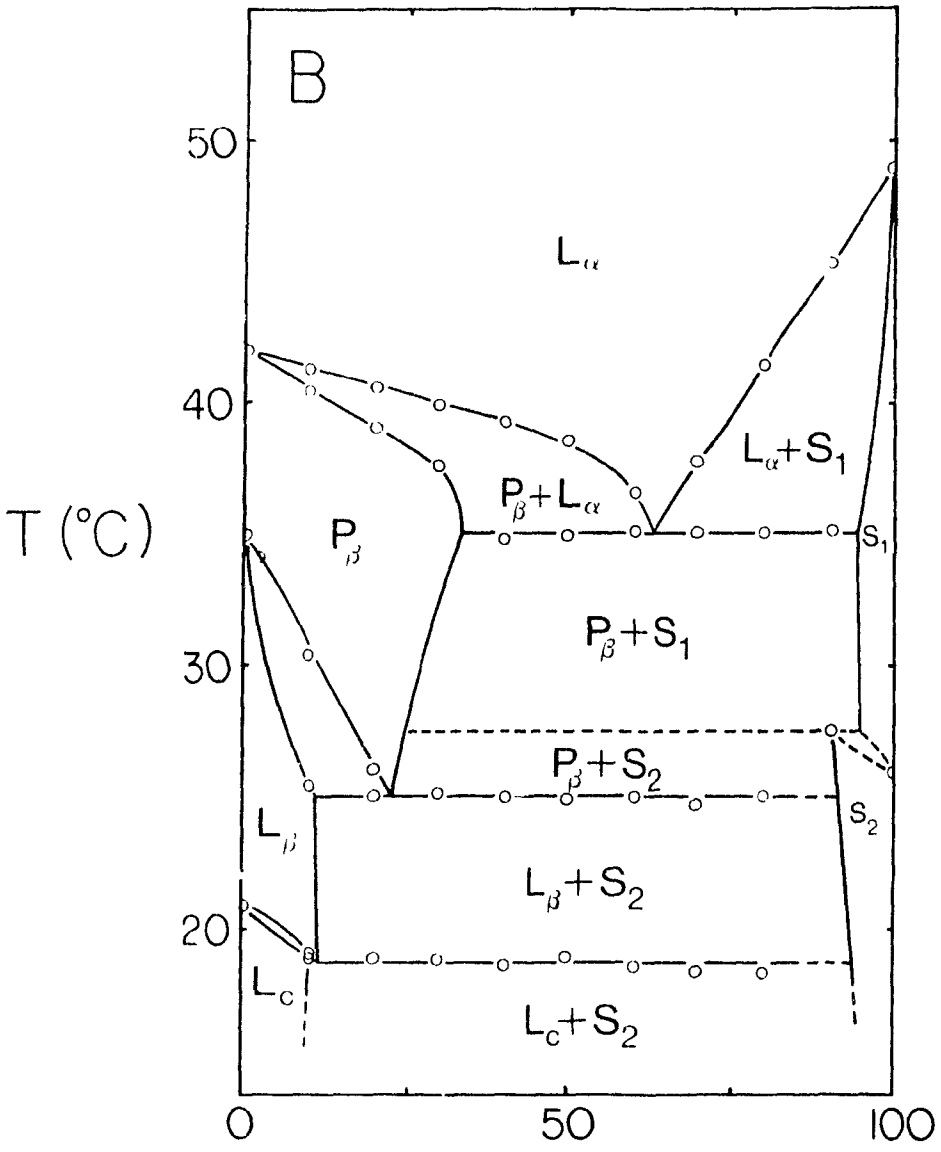
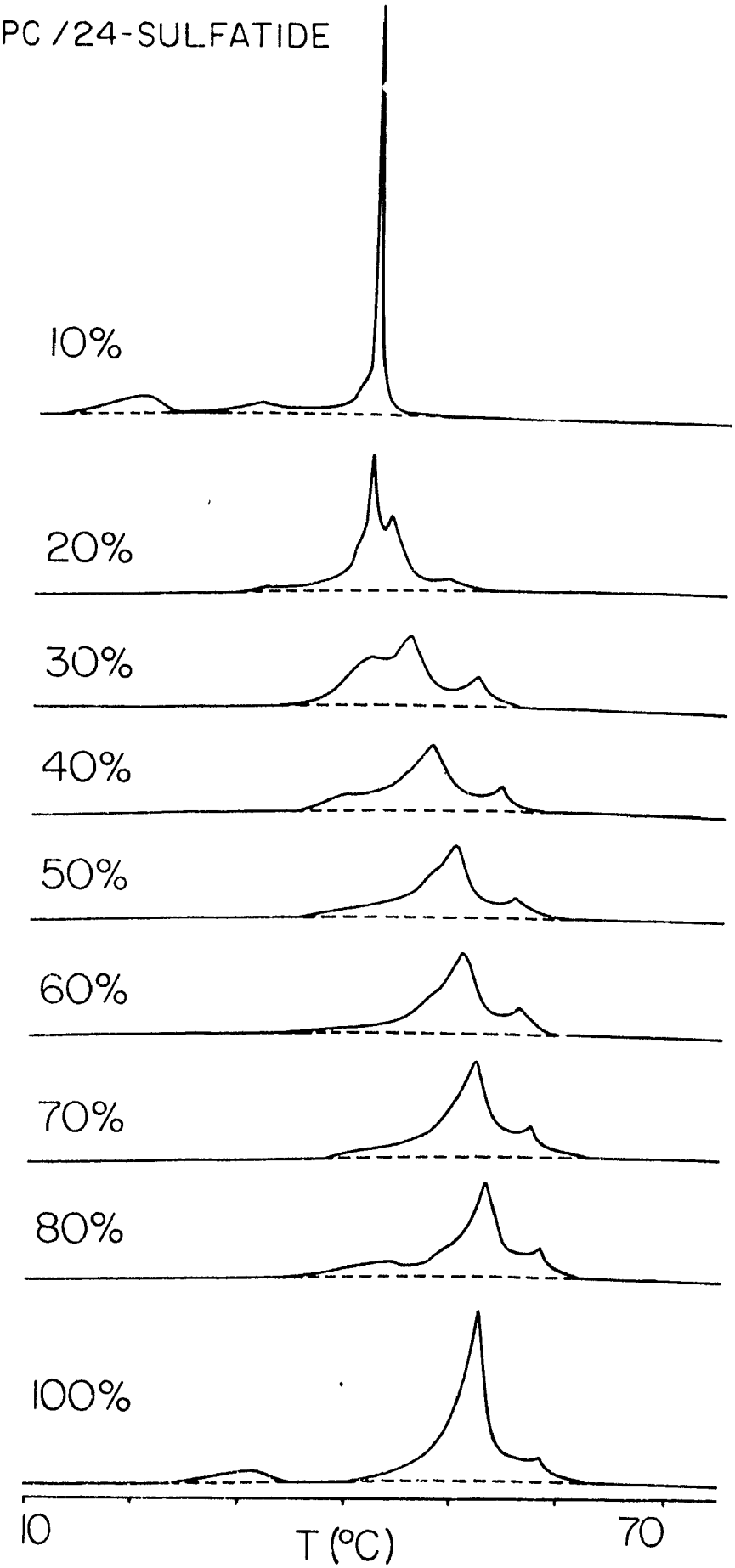

B
50
40
30
20
0
50
100
T (°C)
MOLE % 12/24-PC
L_α
$L_\alpha+S_1$
$P_\beta+L_\alpha$
P_β
S_1
$P_\beta+S_1$
$P_\beta+S_2$
S_2
L_β
$L_\beta+S_2$
L_c
L_c+S_2

Figure 10.

Heating thermograms recorded for mixtures of DPPC plus the indicated molar percentages of 24:0-sulfatide. Samples were dispersed at 60°C and incubated for 60 days at 4°C prior to calorimetry. Other details of sample preparation and calorimetric analysis were as given in Materials and Methods.


DPPC /24-SULFATIDE
10%
20%
30%
40%
50%
60%
70%
80%
100%
10
T (°C)
70

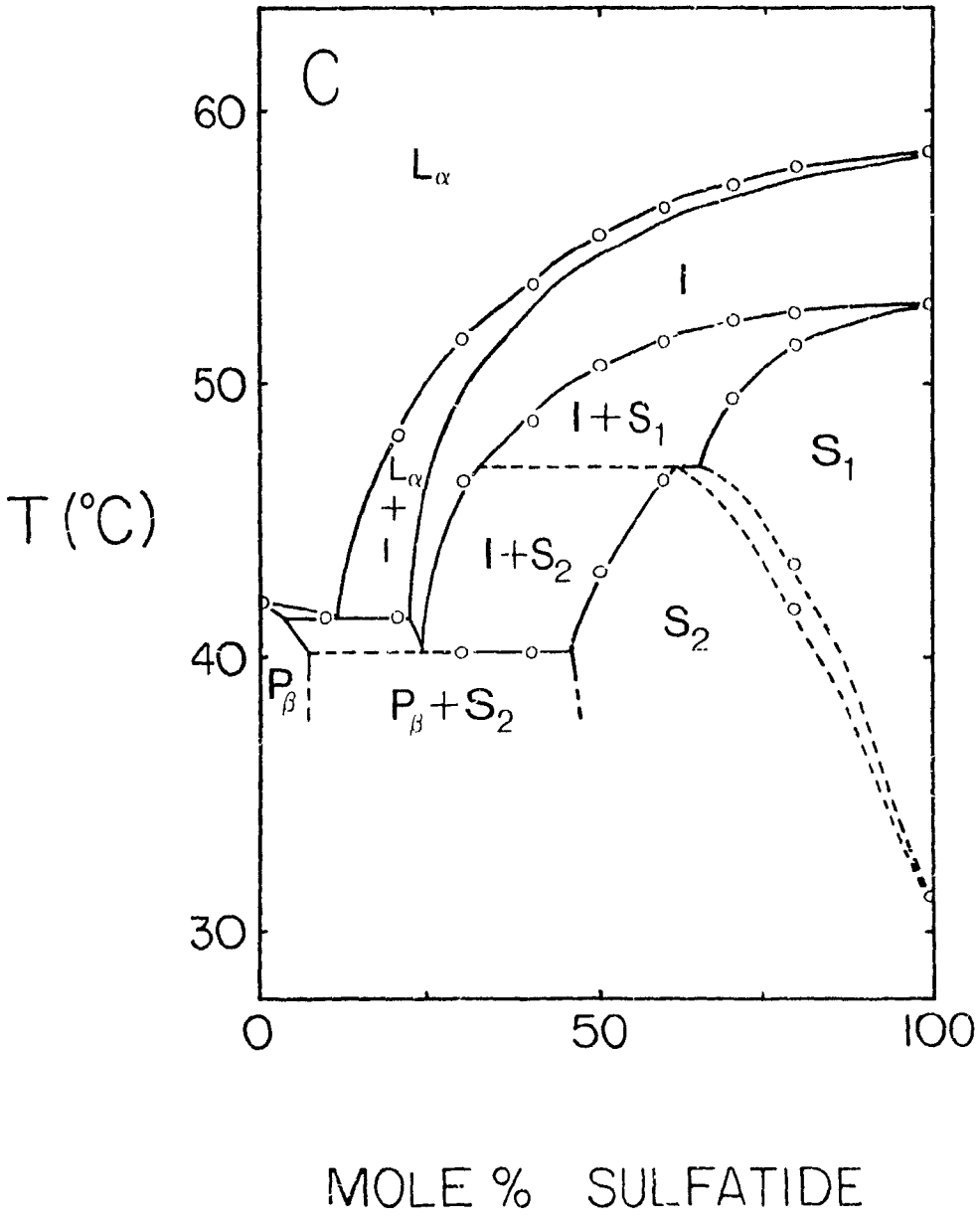
transitions separated by 1-2 °C (not shown). Given the rich polymorphic behavior observed previously for this and other species of sulfatide using other conditions of sample preparation (Boggs et al., 1984, 1988a, 1988b), this behavior is not surprising. An additional small, relatively broad endotherm is seen, with a peak at 41.8°C, in the thermograms of samples of pure N-24:0 sulfatide that are incubated at 4°C for either a few hours or 60 days after dispersal at 65°C. A low-temperature transition has also been reported by Boggs et al. (Boggs et al., 1984) for samples of this lipid dispersed at relatively low salt concentrations.

As increasing amounts of N-24:0 sulfatide are incorporated into DPPC bilayers, both the pre- and the subtransition shift to lower temperatures and become less distinct (Figure 10). In contrast, the main phase transition of DPPC broadens and gradually shifts to higher temperatures as the proportion of N-24:0 sulfatide in the bilayers increases. The lower boundary of the main transition envelope remains near 41°C up to 40 mole% N-24:0 sulfatide but then progressively increases as the molar percentage of sulfatide increases still further. Interestingly, the small upper-temperature transition observed for pure N-24:0 sulfatide is also observed for mixtures containing as little as 20 mole% sulfatide in DPPC.

In Figure 11 is shown the phase diagram inferred from the above calorimetric results for the DPPC - N-24:0 sulfatide system. The presence of multiple upper-temperature transitions for N-24:0 sulfatide complicates substantially the complete assignment of all features in the phase diagram. However, the position of the liquidus curve, and the limits of the phase separation immediately below 40°C, can be assigned fairly accurately. DPPC and N-24:0 sulfatide show a limited region of gel-state immiscibility, extending from <10 mole% to just over 40 mole % sulfatide. The liquidus curve, which was defined from the upper boundary of the high-temperature transition, indicates complete (but not ideal) miscibility of the two lipids in the liquid-crystalline state. The remainder of the phase diagram for this system above 40°C is plotted to rationalize the behavior of the major and the high-temperature transitions in samples containing 20

Figure 11.

Phase diagram deduced from calorimetric data for binary mixtures of DPPC with N-lignoceroyl-3'-sulfogalactocerebroside (N-24 sulfatide). Phases are designated as follows: L_α, liquid-crystalline; P_β', 'ripple' phase formed by DPPC between the pre- and main transitions; L_β', gel phase formed by DPPC between the sub- and pre-transitions; I_1, subgel phase formed by DPPC; S_1, S_2, upper- and lower-temperature solid phases formed by the lignoceroyl species; I_2, intermediate phase formed by 24:0 sulfatide between the main and the highest-temperature transition. Data points plotted represent the onset and completion temperatures, estimated using the procedure of Mabrey and Sturtevant (26), for endothermic events associated with regions of two-phase coexistence in the phase diagram, as well as the peak temperatures measured for endothermic features associated with lines of three-phase coexistence. Phase boundaries that can be reliably assigned from the calorimetric data are indicated by solid lines in the phase diagrams, while boundaries whose position cannot be assigned precisely, or which must be postulated in order to complete the phase diagrams in accordance with the phase rule, are indicated as dashed lines. For clarity, some regions of the phase diagrams are left unlabeled when the phases present can be clearly inferred from the labeling of the adjacent regions.


C
60
50
40
30
T (°C)
L_α
I
L_α + I
I+S_1
S_1
I+S_2
S_2
P_β
P_β+S_2
0
50
100
MOLE % SULFATIDE

mole% to 100 mole% N-24:0 sulfatide, on the assumption that an intermediate phase (designated 'I') intervenes between the gel and the liquid-crystalline states in these samples. The behavior of the lower-temperature transition of N-24:0 sulfatide-rich mixtures is also included in the phase diagram, as phase boundaries related to this transition appear to intersect the solidus curve. The pre- and subtransitions of DPPC are not included in this phase diagram, since they cannot be mapped accurately in samples containing 20 mole% or more N-24:0 sulfatide. However, these transitions do not appear to be involved in determining the position of the solidus curve.

3.5 Discussion:

While the formation of interdigitated lamellar phases in a variety of single-component lipid systems is well-documented, less is known about the potential for such phases to form in lipid mixtures, particularly in mixtures that contain both lipid species that form interdigitated phases in isolation and species that do not. Coexistence of interdigitated and noninterdigitated gel phases has been observed in the dihexadecyl PC/dipalmitoyl PC system (Lohner et al., 1987; Kim et al., 1988), and Mason (1988) has reported intriguing evidence for a limited liquid-liquid phase separation, as well as segregation of interdigitated and noninterdigitated gel phases, in mixtures of distearoyl PC with 1-stearoyl-2-decanoyl PC. It has been suggested (Huang and Mason, 1986; Mason, 1988) that naturally occurring lipid species with highly discrepant hydrocarbon chain lengths, such as very long-chain sphingolipids, may form segregated interdigitated domains at physiological temperatures in mixed-lipid bilayers and even in some biological membranes. However, little direct evidence has been provided to assess this possibility.

Dipalmitoyl PC and 12:0/24:0-PC show complete miscibility in the liquid-crystalline state but extensive phase separation in the gel state, leading to the appearance of a eutectic point. The acyl chains of 12:0/24:0-PC show a mismatch in their effective lengths, quantitated by the parameter ($\Delta C/CL$) defined by Huang and Mason (1986), of almost exactly 0.5. Xu and Huang (1987) have shown that phosphatidylcholines manifesting this degree of acyl chain mismatch form mixed interdigitated bilayers in the gel state. The gel-phase immiscibility observed between 12:0/24:0-PC and DPPC thus presumably reflects the incompatibility of interdigitated and noninterdigitated gel phases, as has been observed for the dihexadecyl PC/DPPC and the distearoyl PC/1-stearoyl-2-decanoyl PC systems (Lohner et al., 1987; Kim et al., 1988; Mason, 1988). A similar conclusion has very recently been reported by Lin and Huang (1988) to explain the eutectic behavior observed in mixtures of dimyristoyl PC and 1-stearoyl-2-caproyl PC.

The phase diagram determined for the DPPC/N-24:0 cerebroside system reveals extensive phase separation in the gel, but not in the liquid-crystalline state. In view of the results discussed above, it might seem reasonable to conclude that this extensive gel-phase separation is primarily attributable to the incompatibility of a noninterdigitated PC-rich gel phase and an interdigitated gel phase rich in N-24:0 cerebroside. However, this conclusion can be questioned on several grounds. First, Ruocco et al. (1983) have reported that mixtures of DPPC and N-palmitoyl cerebroside exhibit phase separation in the gel state over a wide range of compositions. Other PC-cerebroside mixtures, including mixtures of egg PC, DPPC or 1-palmitoyl-2-oleoyl PC with bovine brain cerebroside, have also been found to exhibit extensive phase separation in the gel state (Maggio et al., 1985; Curatolo, 1986; Runow and Levin, 1988, Johnston and Chapman, 1988). The marked tendency of N-24:0 cerebroside to phase-separate from DPPC in the gel state may thus be determined more by its headgroup structure than by its potential to form an interdigitated solid phase. Second, Reed and Shipley (1987) have reported evidence that N-24:0 cerebroside forms partially interdigitated solid phases, in which neither acyl chain extends completely across the lipid bilayer, rather than mixed interdigitated solid phases, in which the longer acyl chain would span the entire thickness of the hydrocarbon region. It is thus difficult to interpret the phase separation in the DPPC/N-24:0 cerebroside system as analogous to that observed in mixtures of DPPC with 12:0/24:0-PC, where the gel phase of the lignoceroyl component is expected to adopt a mixed interdigitated structure. The limited solid-solid phase separation that we observe in mixtures of DPPC with N-24:0 sulfatide, which forms a mixed interdigitated gel phase (Boggs et al., 1988), likewise cannot be attributed purely to the incompatibility of interdigitated and noninterdigitated gel phases. Rintoul et al. (1988) have reported that mixtures of dielaidoyl PC with natural brain sulfatide, which does not form an interdigitated gel phase (Ruocco and Shipley, 1986), show a solid-phase separation that is similar in extent to that observed here for the DPPC/N-lignoceroyl sulfatide system.

In the liquid-crystalline state 12:0/24:0-PC, N-24:0 cerebroside and N-24:0 sulfatide are all fully miscible with DPPC. Moreover, the general behavior of the liquidus curve in the phase diagram for the DPPC/N-24:0 cerebroside system is very similar to that observed previously in the phase diagrams for other PC-cerebroside mixtures (Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986; Bunow and Levin, 1988; Johnston and Chapman, 1988), allowing for differences in the transition temperatures of the lipid components in the different systems. It is clear that the N-lignoceroyl lipid species studied here, with highly discrepant hydrocarbon chain lengths, can readily accomodate to the fluid matrix formed by a symmetric-chain lipid such as DPPC. Grant and co-workers (1987, 1988) have presented evidence that the long acyl chains of several spin-labeled N-lignoceroyl glycosphingolipids extend across the bilayer midplane, penetrating substantially into the opposing monolayer, when low mole percentages of the sphingolipid labels are incorporated into bilayers composed of symmetrical phospholipids. It would appear from the results presented here that such interdigitation allows good mixing of lipids with symmetrical and asymmetrical hydrocarbon chains in the liquid-crystalline state. This result agrees with the findings of Grant and co-workers (1987, 1988) that spin-labeled N-lignoceroyl glycosphingolipids reveal no special tendency to cluster when present at low mole fractions in bilayers of lipids such as egg PC and dimyristoyl PC.

The phase diagrams presented in this study, when compared to those reported previously for other phospholipid/ glycosphingolipid mixtures (Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986; Bunow and Levin, 1988; Johnston and Chapman, 1988; Rintoul et al., 1988), provide no evidence that N-lignoceroyl sphingolipids are markedly more prone to segregate laterally from liquid-crystalline phospholipids than are the corresponding sphingolipids with shorter acyl chains. These results raise some question concerning the suggestion that the primary biological function of the very long N-acyl chains of some sphingolipids is to promote the lateral segregation of sphingolipid-enriched domains in the membrane plane. It is more likely, in view of the present results, that very long-chain

sphingolipids may serve to confer special physical properties (e.g., greater stability or enhanced mechanical coupling between the bilayer leaflets [Schmidt et al., 1978]) to the membrane as a whole, or possibly to sphingolipid-enriched domains that may segregate in the membrane plane on the basis of properties other than the length of the lipid acyl chains.

CHAPTER FOUR: General Discussion

The physical interactions between lipid molecules have customarily been examined using simple model systems, which contain either a single lipid species or two defined lipid species in the case of mixing experiments. Such studies have contributed much information toward our current understanding of lipid physical behaviour. It is important to note, however, that model membrane systems, like all scientific models, have inherent limitations. For example, if two lipids are found to mix nearly ideally in a binary model system, it is likely that they will exhibit similar behaviour when part of a more complex system, such as the multicomponent lipid bilayer of a biological membrane. By contrast, if the lipids exhibit phase separation in a simple binary system, it may be difficult to automatically extrapolate this behaviour to that found in a biological membrane where the presence of many lipid species may mitigate the tendency toward segregation of well-defined phases. Likewise, conclusions obtained from studies of the physical behaviour of lipid probes (e.g., fluorescent-labelled or spin-labelled lipids), must be interpreted in the light of proper controls which eliminate possible 'probe artifacts'. In general however, when applied with suitable controls, model membranes and membrane probes have been extremely useful in the study of membrane structure and function.

One of the classical approaches to the study of simple binary mixtures is high-sensitivity differential scanning calorimetry. This technique involves the measurement of the heat capacity of a lipid sample relative to a reference, as the temperature of the system is scanned in the range of interest. In this fashion, it is possible to obtain detailed information about the enthalpies and entropies of mixing of the lipid species present in the sample. Unfortunately, because of the complexity of the information obtained from such studies, it is very difficult to analyze samples containing more than two or three total components (e.g., H_2O and two lipid species), let alone natural membranes. Another, though less fundamental problem in extrapolating from calorimetric studies of simple model systems to biological membranes arises from the fact that the samples typically examined in calorimetric studies are not identical in morphology to

natural membranes (i.e., unilamellar structures with very large radii of curvature). The samples used in most calorimetric studies, for example, are usually prepared as multilamellar vesicles which allow the potential for interactions between the lipids of adjacent lamellae that are not normally present in biological membranes.

The use of fluorescent lipid probes to study membrane systems has become an accepted and widely used technique. These probes consist of a lipid molecule labeled on either its polar headgroup or on one of its hydrocarbon chains with a fluorescent group. Since the lipid structure is considerably altered with the addition of the fluorescent group, it is possible that the behaviour exhibited by such a probe may be due to this group and thus, may not be similar to that of the unlabelled species. It is therefore important to rule out such a possibility by examining the behaviour of two or more probes; if the different probes are found to have similar behaviour, one can conclude that the fluorescent label has little effect.

It has been suggested, based on data obtained from model studies, that attractive forces between the headgroup and/or acyl chain portions of certain lipids may favour the formation of separate lipid domains in multicomponent liquid-crystalline bilayers (Dragsten et al., 1981; Spiegel et al., 1985, Metcalf et al., 1987; van Meer and Simons, 1986; Gordon et al., 1987; Yechiel and Edidin, 1987). Such domains have been theorized to participate in a number of membrane functions, such as ligand binding to the cell surface (Quarfe-Casas, 1973, Haywood, 1974; Revesz and Greaves, 1975; Saffman and Delbruck, 1975; Sedlacek et al., 1976; Mullin et al., 1976; Holmgren et al., 1980; Critchley, et al., 1981; Reed et al., 1987; Masserini et al., 1988), the formation of membranes with unusual morphology (Curatolo, 1987), and the enhancement of membrane stability (Schmidt, et al., 1978). Arguably, the lipid species most likely to exhibit lateral segregation to form such domains in biological membranes are the glycosphingolipids. These lipids possess substantial hydrogen-bonding capacity in their polar headgroups (Boggs, 1987), and a large fraction of these lipids found in natural membranes carry highly asymmetric hydrocarbon chains (van

Deenen and de Gier, 1974; Abe and Norton, 1979). To date, however, few studies have systematically examined the thermotropic behaviour of glycosphingolipids in simple mixtures with phospholipids.

Chapter 3 of this thesis describes the use of a traditional approach to examine the mixing of symmetrical phospholipids with asymmetric sphingo- and phospholipids. High sensitivity differential scanning calorimetry was employed to examine the mixing of DPPC with three asymmetric lipid species: N-lignoceroyl cerebroside and sulfatide, and 1-lauroyl-2-lignoceroyl phosphatidylcholine. The long-chain sphingolipid species are representative of naturally occurring sphingolipids while the long-chain PC, although not naturally occurring, was examined as a 'control', having the same polar headgroup as DPPC but a degree of hydrocarbon chain asymmetry comparable to that of the N-lignoceroyl sphingolipids. All three species were found to be highly miscible with DPPC in the liquid-crystalline state, indicating that the fluid bilayer matrix can readily accommodate lipids bearing highly asymmetric hydrocarbon chains. These results, therefore, shed some doubt on the above suggestion that the function of asymmetric lipids, especially long-chain sphingolipids, in natural membranes may be linked to their ability to promote the segregation of sphingolipid-rich domains.

An alternative and relatively novel approach to calorimetric mixing experiments such as those just described, involves the study of lipid probe partitioning between different bilayer environments, as described in Chapter 2. It is obvious, given the diversity of lipid structures found in natural membranes, that not all the different lipid species found in a given membrane may interact 'ideally', i.e. with equivalent energies. The important point addressed by the work described in chapter 2 was not that a given lipid species may show some discrimination in its interactions with other types of lipids, but rather the magnitude of this selectivity. I have found that different phospho- and sphingolipid classes differ only modestly in their relative affinities for 'hydrogen-bonding' vs. 'non-hydrogen-bonding environments. Previous calorimetric studies had suggested that lipids that can act as both hydrogen-bond donors

and acceptors (e.g., amino phospholipids) may interact in a highly nonideal manner with lipids that can only accept hydrogen bonds, such as the choline phospholipids (Shimsick and McConnel, 1973; Wu and McConnel, 1975; Mabrey and Sturtevant, 1976; Arnold et al., 1981; Ruocco et al., 1983, Maggio et al., 1985, Curatolo, 1986, 1987; for review, see Boggs, 1987). Similarly, it was found in this study that while the order of the relative affinities of different lipids for cholesterol-containing vs. cholesterol-free environments agreed with the majority of previous reports (van Dijck et al., 1976, 1979; Demel et al. 1977; Wattenberg et al., 1983; Fugler et al., 1985; Yeagle and Young, 1986), the degree of variation in this selectivity with lipid headgroup structure was considerably smaller than most previous reports would suggest. These results indicate that lipids with different headgroups may intermix in the liquid crystalline phase more nearly ideally than previously thought. Several reasons for this discrepancy between our conclusions and those of previous studies have been discussed in Chapter 2 and will not be presented here.

On the basis of the above data, taken together with other previously reported data for a variety of binary lipid systems, it appears unlikely that spontaneous segregation of lipid domains, on the basis of selective headgroup or acyl chain interactions among particular lipid species, is likely to occur in bilayer membranes with the compositions typical of animal cells. In order to prove or disprove this hypothesis conclusively, of course, it is necessary to look for the existence of such behaviour in more complex membrane systems, including biological membranes. While the methodologies needed to address this latter question are poorly developed at present, I discuss below some feasible future approaches by which this problem could be addressed.

The technique of fluorescent probe partitioning is particularly suited to the study of lipid clustering behaviour in natural membranes because it enables one to study far more complex membrane systems (including biological membranes) than could be studied using the very simple (one-to-three-component) systems that are amenable to

study by more traditional techniques such as calorimetry. One can thus envision several experiments using different types of phospho- and sphingolipid probes to address the possibility of lipid 'domain' formation in biological membranes. For example, exchangeable self-quenching glycosphingolipid and phospholipid probes could be synthesized which could readily partition into biological membranes (e.g., erythrocyte ghosts) in which their fluorescence would be sensitive to the lateral distribution (e.g., dispersed vs. 'clustered') of the probe molecules. From the equilibrium fluorescence intensity value measured for probes with different headgroups, one would be able to determine, for example, if glycosphingolipid probes were more prone to laterally aggregate (i.e., would show a greater degree of self-quenching) than phospholipid probes bearing the same fluorescent moiety. A related procedure would involve the labelling of natural membranes with two different lipid probes, the second fluorescent lipid would quench the first with an efficiency dependent on the lateral distribution of the two types of probe.

In a third approach, one could measure the relative partition coefficients of several lipid probes between natural bilayer environments of different lipid composition. These measurements would provide a means to determine which probes exhibit nonideal mixing with the bulk membrane lipids, or whether there is a possibility of formation of laterally segregated domains of certain species (e.g., through preferential association with certain membrane proteins). Finally, some recent work (de Bony et al., 1989) involving anthracene-labelled lipids also shows promise in the study of lipid mixing in biological membranes. These photodimerizing lipid probes can be incorporated into different natural membranes, exposed to ultra-violet light and the resulting number of photodimers counted giving a measure of the clustering behaviour of the different lipid species in a given membrane.

In a similar fashion to the fluorescent studies outlined above, spin-labelling studies could also be used to look for lipid-clustering behaviour in biological membranes. Grant and coworkers (Grant et al., 1987; Melhorn et al. 1988) have recently used spin-labelled

glycosphingolipid species in order to determine whether or not glycosphingolipid-rich phases exist in simple phospholipid bilayers, and it is possible to imagine how more complex systems could be studied by these same types of approaches. For example, one could synthesize exchangeable spin-labelled lipid probes and allow them to partition in different biological membranes as suggested above, and look for evidence of spin-exchange broadening, indicating the presence of probe clustering behaviour.

As indicated in the introduction of this thesis, natural membranes contain a great number of different lipid species. It seems unlikely that such diversity could exist without biological advantage, and thus, the different lipid classes must have somewhat different roles to play in maintaining membrane structure and optimal membrane function. It seems likely, on the basis of the experiments I have presented here, that these roles do not involve the thermodynamically spontaneous formation of segregated lipid domains in multicomponent lipid bilayers such as those found in biological membranes. Therefore, I suggest that the function of those lipid species whose structures diverge greatly from the saturated/unsaturated motif characteristic of most membranes (e.g., the sphingolipids) lies elsewhere. It is possible that lipids bearing asymmetric acyl chains, such as naturally occurring sphingolipids, may have significant effects on lipid-protein interactions. Membrane proteins cause a large perturbation of the membrane structure on insertion into the bilayer (Gullis and De Kruijff, 1979, Israelachvili, 1977). Some evidence suggests that lipids with irregular geometries such as those possessing asymmetric chains could pack around the protein and thus maintain the continuity of the bilayer (Gerritsen et al., 1979). Another role of asymmetric lipids in natural membranes involves the mechanical stabilization of lipid bilayers through acyl interdigitation in the centre of the bilayer (Schmidt et al., 1978).

Since the publishing of the Singer and Nicholson fluid-mosaic model of biological membranes (1972), an enormous amount of work has been performed on elucidating the structure and function of membranes

and of individual membrane components. This previous work has shown that the functional role of the lipid component of biological membranes is far more complex than originally envisioned. The work presented in this thesis argues against one intriguing potential role for lipid diversity in biological membranes, namely the spontaneous creation of laterally segregated domains, with distinct compositions, in biological membranes.

CHAPTER FIVE: References

Abe, T. and Norton, W.T. (1979) J. Neurochem 32, 823-832.

Abrahamsson, S., Dahlen, B., Lofgren, H., Pascher, I. and Sundell, S. (1977) in Structure of Biological Membranes (Abrahamsson, S. and Pascher, I., ed.), pp. 1-23, Plenum Press, New York.

Albon, N., and Sturtevant, J.M. (1978) Proc Natl. Acad. Sci. U.S.A 75, 2258-2260

Arnold, K., Losche, A. and Gawrisch, K. (1981) Biochim. Biophys. Acta 645, 143-148.

Bach, D., Bursuker, I., Eibl, H. and Miller, I.R. (1978) Biochim. Biophys Acta 514, 310-319.

Barenholtz, Y., Suurkuusk, J., Mountcastle, D., Thompson, T.E., and Biltonen, R.L. (1976) Biochemistry 15, 2441-2447.

Barenholtz, Y., and Gatt, S. (1982) in Phospholipids (Hawthorne, Ansell eds.), pp. 129-177, Elsevier Biomedical Press, The Netherlands.

Barenholz, Y., and Thompson, T.E. (1980), Biochim. Biophys. Acta 604, 129-158

Barton, P.G. and Gunstone, F.D. (1975) J. Biol. Chem. 250, 4470-4476.

Berridge and Irvine (1984) Nature 312, 315-320.

Blume, A., Wittebort, R.J., Das Gupta, S.K. and Griffin, R.G (1982), Biochemistry 21, 6243-6253.

Blume, A. and Eibl, H. (1981) Biochim. Biophys. Acta 640, 609-618

Blume, A. and Griffin, R.G. (1982) Biochemistry 21, 6230-6242.

Blume, A. and Eibl, H. (1979) Biochim. Biophys. Acta 558, 13-21.

Boggs, J.M. (1980), Can. J. Biochem. 58, 755-770.

Boggs, J.M., and Rangaraj, G. (1985) Biochim. Biophys. Acta 816, 221-233

Boggs, J.M., Koshy, K.M., and Rangaraj, G. (1984) Chem. Phys. Lipids 36, 65-89.

Boggs, J.M., Koshy, K.M., and Rangaraj, G. (1988b) Biochim. Biophys. Acta 938, 361-372.

Boggs, J.M., Koshy, K.M., and Rangaraj, G. (1988a) Biochim. Biophys. Acta 938, 373-385.

Boggs, J.M., (1987) Biochim. Biophys. Acta 906, 353-404.

Boggs, J.M., and Mason, J.T. (1986) Biochim. Biophys. Acta 863, 231-242.

Boggs, J.M., Rangaraj, G., Moscarello, M.A. and Koshy, K.M. (1985) Biochim. Biophys. Acta 816, 208-220.

Boggs, J.M., Stamp, D. and Moscarello, M. (1986) Biochim. Biophys. Acta 863, 231-240.

Bonn, T.T., and Hui, S.W. (1983) Biochim. Biophys. Acta 731, 177-184.

Braganza, L.F. and Worcester, D.L. (1986) Biochemistry 25, 2591-2596.

Brauner, J.W., and Mendelsohn, R. (1986) Biochim. Biophys. Acta 861, 16-24

Browning, J.L. (1981b) Biochemistry 20, 7133-7143.

Browning, J.L. (1981a) Biochemistry 20, 7123-7133.

Bunow, M.R. (1979) Biochim. Biophys. Acta 574, 542-546.

Bunow, M.R. and Levin, I.W (1980) Biophys. J. 32, 1007-1022.

Bunow, M.R., and Levin, I.W. (1988) Biochim. Biophys. Acta 577-586.

Burnell, E., Van Alphen, L., Verkleij, A. and De Kruijff, B (1980) Biochim. Biophys. Acta. 597, 492-501.

Caffrey, M., and Feigenson, G.W. (1984) Biochemistry 23, 323

Calhoun, W.I. and Shipley, G.G. (1979) Biochemistry 18, 1717-1722.

Cestaro, B., Cervato, G., Marchesini, S., Viani, P., Pistolesi, L., and Oliva, C. (1983) Chem. Phys. Lipids 33, 251-262.

Ceve, G., Watts, A., and Marsh, D (1981) Biochemistry 20, 4955-4965.

Chapman, D., Urbina, J and Keough, K.M. (1974) J. Biol. Chem 249, 2512-2521.

Chen, S.C., and Sturtevant, J.M. (1981) Biochemistry 713-718

Cheng, W.H. (1980), Biochim. Biophys. Acta 600, 358-366.

Clowes, A.W., Cherry, R.J. and Chapman, D (1971) Biochim Biophys Acta 249, 301-317.

Cockcroft and Gomberts (1985) Nature 314, 534-536

Cohen, R., Barenholz, Y., Gatt, S., and Dagan, A. (1984) Chem. Phys. Lipids 35, 371-384.

Comfurius, P. and Zwaal, R.F.A., (1977) Biochim. Biophys. Acta 488, 36-42.

Coorssen, J.P. and Rand, R.P. (1989) Biochemistry 26, 39-45.

Correa-Freire, M.C., Barenholz, Y. and Thompson, T.E. (1982) Biochemistry 21, 1244-1248.

Correa-Freire, M.C., Freire, E., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1979) Biochemistry 18, 442-445.

Critchley, D.R., Megnami, J.J. and Fishman, P.H. (1981) J. Biol Chem 256, 8724-8731.

Cuatrecasas, P. (1973) Biochemistry 12, 3547-3558; 3558-3566.

Cullis, P.R., and Hope, M.J (1978) Nature, 271, 672-674.

Cullis, P.R. and de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399-420.

Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207-218.

Curatolo, W (1986) Biochim. Biophys. Acta 861, 373-376.

Curatolo, W. (1987) Biochim. Biophys. Acta 906, 111-136.

Curatolo, W. (1982) Biochemistry 21, 1761-1764.

Curatolo, W., Bali, A., and Gupta, C.M. (1982) Biochim. Biophys. Acta 690, 89-94.

Curatolo, W., Bali, A., and Gupta, C.M. (1985) J. Pharm. Sci. 74, 1255-1258.

Curatolo, W. and Jungalwala, F.B. (1985) Biochemistry 24, 6608-6613.

Curatolo, W. and Neuringer, L.J. (1986) J. Biol. Chem. 261, 17177-17182.

Davis, P.J. and Keough, K.M.W. (1983) Biochemistry 22, 6334-6340.

Davis, P.J., Fleming, B.D., Coolbear, K.P., and Keough, K.M.W. (1981) Biochemistry 20, 3633-3636.

de Bony, J., Lopez, A., Gilleron, M., Welby, M., Lancelle, G., Rousseau, B., Beaucourt, J.P., and Tocanne, J.F. (1989) Biochemistry 28, 3728-3737.

Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1-10

Demel, R.A., Jansen, J.W.C.M., van Dijck, P.W.M. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1-10

Dragsten, P.R., Blumenthal, R. and Handler, J.S. (1981) Nature 294, 718-722.

Eibl, H. (1982) in Membrane Fluidity in Biology Vol II (Aloia, R.C., ed.) pp. 217-236, Academic Press, New York.

Eibl, H. and Blume, A. (1979) Biochim. Biophys. Acta 553, 476-488

Epand, R.M., and Bottega, R. (1988) Biochim. Biophys. Acta 944, 144-154

Eriksson, P., Rilfors, L., Lindblom, G. and Arvidson, LG. (1985) Chem. Phys. Lipids 37, 357-371.

Estep, T.N., Mountcastle, D.B., Biltonen, R.L. and Thompson, T.E. (1978) Biochemistry 17, 1984-1989.

Estep, T.N., Freire, E., Anthony, K.F., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1981) Biochemistry 20, 7115-7118.

Evans, W.H and Hardison, W.G M. (1985) Biochem J. 232, 33-36.

Ferguson, G W (1989) Biochemistry 28, 1270-78.

Ferrell, J.E., Jr., Lee, K.-J. and Huestis, W. (1985) Biochemistry 24, 2857-2864

Fishman, P H. (1986) Chem. Phys. Lipids 42, 137-151.

Florine K I. and Ferguson, G.W. (1987) Biochemistry 26, 1757-1768

Frank, V., Barenholz, Y., Lichtenberg, D. and Thompson, T.E. (1983) Biochemistry 22, 5647-5651.

Freire, E., Bach, D., Correa-Freire, M., Miller, I. and Barenholz, Y (1980) Biochemistry 19, 3662-3665.

Fugler, L., Olejan, S. and Rittman, R. (1985) J Biol Chem 260, 4098-4101.

Gaffney, P J. and Chen, S.-C. (1977) Methods Memb. Biol. 8, 291-358.

Gigue, J., Stamatatos, I., Diacovo, I., Hui, S.W., Yeagle, P.L., and Silvius, J.R. (1985) Biochemistry 24, 4400-4408.

Galla, H.-J., and Sackhman, E. (1975) Biochim. Biophys. Acta 401, 509- 529.

Gardam, M.A., Itovitch, J.J., and Silvius, J.R. (1989) Biochemistry 29, 884-893.

Gardam, M., and Silvius, J.R. (1989) Biochim. Biophys. Acta 980, 319-325.

Gerritsen, W.J., van Zoelen, E.G.G., Verkleij, A.M., de Kruijff, B. and van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 248-259.

Gordon, L.M., Looney, F.D., and Curtain, C.C. (1987) Biochim. Biophys. Acta 898, 202-213.

Graham, J., Gagné, J., and Silvius, J.R. (1985) Biochemistry 24, 7123-7131.

Grant, C.W.M., Mehlhorn, I.E., Florio, F., and Barber, F.R., (1987) Biochim. Biophys. Acta 902, 169-177

Haines, T.H. (1971) Prog. Chem. Fats and other Lipids 11, 299-345

Hartmann, W., Galla, H.-J., and Sackmann, E. (1977) FEBS lett 78, 169.

Hauser, S., Pascher, I., Pearson, R.H., and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21-51.

Haverstick, D.M. and Gaser, M. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4475-4479.

Haverstick, D.M. and Gaser, M. (1988) J. Cell Biol 106, 1885-1892

Haywood, A.M. (1974) J. Mol. Biol 83, 427-436.

Heminger, H. -J., Kandutsch A.A., Chen, H.W., (1976) Nature 263, 515-517.

Hinz, H -J., Six, L., Ruess, K.-P., and Liefländer, M. (1985) Biochemistry 24, 806-813.

Hitchcock P.B., Mason, R., Thomas, K.M and Shipley, G.G. (1974) Proc. Natl. Acad. Sci. U.S.A 71, 3036-3040.

Hoekstra, D (1982) Biochemistry 21, 1055-1061

Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P and Strannegard, O (1980) Proc Natl. Acad. Sci. U.S.A 77, 1947-1950

Holub, B.J and Kuksis, A (1978) Adv. Lipid Res. 16, 1-125.

Hresko, R.C., Sugar, I.P., Barenholz, Y. and Thompson, T.E. (1987) Biophys. J. 51, 727-733.

Huang, C and Levin, I.W. (1983) J. Phys. Chem. 87 1509-1513.

Huang, C., Mason, J.T., and Levin, I.W. (1983) Biochemistry 22, 2775-2780

Huang, C and Mason, J.T (1986) Biochim. Biophys. Acta 864, 423-470

Huang, C., Lapides, J.R. and Levin, I.W. (1982) J. Am. Chem Soc. 104, 5926-5930.

Hui, S.W. and Huang, C.-h. (1986) Biochemistry 25, 1330-1335.

Hui, S.W., Boni, L.T., Stewart, T.P., and Isac, T. (1983) Biochemistry 22, 3511.

Hui, S.W., Mason, J.T., and Huang, C. (1984) Biochemistry 23, 5570-5577.

Hui, S.W., Bon, L.T., Stewart, T.P., and Issac, T. (1983) Biochemistry 22, 3511-3516.

Hui, S.W. and He, N. (1983) Biochemistry 22, 1159-1164

Israelachvili, J.N. (1977) Biochim Biophys Acta 469, 221-225.

Israelachvili, J.N., Mitchell, D.J and Ninham, B.W (1976) J Chem Soc, Faraday Trans. II 72, 1525-1568.

Ito, T and Ohnishi, S-i, (1984) Biochim Prophys Acta 352, 29-37

Jackson M., Johnston, D.S., and Chapman, D (1988) Biochim Biophys Acta 944, 497-506

Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152-161

Johnston, D.S., and Chapman D (1988) Biochim Biophys. Acta 949 603-614.

Keough, K.M.W., and Davis, P.J (1980) Can. J Biochem 58, 851-858

Keough, K.M.W. and Davis P.J. (1979) Biochemistry 18, 1453-1459

Keough, K.M.W. and Davis, P.J (1984) in Membrane Fluidity (M Kates and L. Manson, eds.), Plenum Press, N.Y., pp 55-97

Kim, J.T., Mattai, J. and Shipley, G.G. (1988) Biochemistry 26, 6599-6603

Kishimoto, Y. (1975) Chem. Phys. Lipids 15, 33-36.

Kleeman, W. and McConnell, H.M. (1976) Biochim. Biophys. Acta 345, 220-230

Knoll, W., Schmidt, G., Ibel, K. and Sackmann, E. (1985) Biochemistry 24, 5240-5246.

Koshy, K.M and Boggs, J.M. (1982) Lipids 17, 998-1000

Koshy, K.M., and Boggs, J.M. (1983) Chem. Phys Lipids 34, 41-53.

Kouaouci, R., Silvius, J.R., Graham, I., and Pézolet, M. (1985) Biochemistry 24.

Kovnova, R.E., Kunnenreich, H.I., Tenchov, B.G., and Hinz, H.-J. (1988) Biochemistry 27, 4612-4619.

Kubesch, P., Boggs, J.M., Luciano, I. Maass, G. and Tummler, B. (1987) Biochemistry 26, 2139-2149

Ladbrooke, B.D and Chapman, D (1969) Chem Phys. Lipids 3, 304-367.

Lange, Y., D'Alessandro, J. and Small, D.M. (1979) Biochim Biophys. Acta 556, 488-498

Lee, A.G (1975) Prog. Biophys Mol. Biol. 28, 3-56.

Lee, A.G (1975) Biochim. Biophys. Acta 413, 11-23.

Lee, A.G., (1977), Biochim. Biophys. Acta 472, 285-344.

Lentz, B.R., Barrow, D.A. and Hoechli, M. (1980) Biochemistry 19, 1943-1954.

Lentz, B.R., Hoechli, M. and Barenholz, Y. (1981) Biochemistry 20, 6803-6809.

Leventis, R., Diacovo, T., and Silvius, J.R. (1987). Biochemistry 26, 3267-3276.

Levin, I.W., Thompson, T.E., Barenholz, Y., and Huang, C. (1985) Biochemistry 24, 6282-6286.

Lin, H.-n. and Huang, C.-h. (1988) Biochim. Biophys. Acta 946, 178-184.

Lohner, K., Schuster, A., Degovics, G., Muller, K. and Laggner, P. (1987) Chem. Phys. Lipids 44, 61-70.

Lowry, R.I. and Tinsley, I.J. (1974) Lipid. 9, 491-492.

Luna, E.J. and McConnell, H.M. (1978) Biochim. Biophys. Acta 509, 462-473.

Luna, E.J., and McConnell, H.M. (1977) Biochim. Biophys. Acta 470, 303-316.

Lutz, H.A., Shih-Chun, I. and Pelel, J. (1977) J. Cell Biol. 73, 548-560.

Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3862-3866.

Mabrey, S. and Sturtevant, J.M. (1976) in Methods in Membrane Biology (Korn, E.F., ed.), Vol. 9, pp. 237-274, Plenum Press, New York.

Mabrey, S., Mateo, P.L. and Sturtevant, J.M. (1978) Biochemistry 17, 2462-2468.

MacDonald, R.C., Simons, S.A. and Baer, E. (1976) Biochemistry 15, 885-891

Maggio, B., Ariga, T., Sturtevant, J.M and Yu, R.K. (1985) Biochim. Biophys. Acta 818, 1-12.

Maggio, B., Fidelio, G.D., Cumar, R.A. and Yu, R.K. (1986) Chem. Phys. Lipids 42, 49-63.

Muntsch, H.H., Martin, A. and Cameron, D.G. (1981) Biochemistry 20, 3138-3145.

Mason, J.T. (1988) Biochemistry 27, 4421-4429.

Mason, J.T., Broccoli, A.V., and Huang, C. (1981) Anal. Biochem. 113, 96-99

Mason, J.T., Huang, C., and Biltonen, R.L. (1981) Biochemistry 20, 6086-6092

Mosserini, M., Palestini, P., Venerando, A. Fiorilli, A., Acquotti, D. and Tettamanti, G. (1988) Biochemistry 27, 7973-7978

Massey, J.B., Gotto, A.M and Pownall, H.J. (1982b) Biochemistry 21, 3530-3536

Massey, J.B., Gotto, A.M and Pownall, H.J. (1982a) J. Biol. Chem. 257, 5444-5448.

Mattai, J., Sripada, P.K., and Shipley, G.G. (1987) Biochemistry 26, 3287-3297

Matubayasi, N., Shigematsu, T., Ishara, T., Kamaya, H., and Ueda, I. (1986) J. Memb. Biol. 90, 37-42.

Maulik, P.R., Atkinson, D., and Shipley, G.G. (1986) Biophys. J. 50, 1071-1077.

McDaniel, R.V., McIntosh, T.J. and Simon, S.A. (1983) Biochim. Biophys. Acta 731, 97-108.

McElhany, R.N. (1984) Biochim. Biophys. Acta 30, 229-259.

McIntosh, T.J., Simon, S.A., Ellington, J.C., and Porter, N.A. (1984) Biochemistry 23, 4038-4044.

Mehlhorn, I.E., Florio, L., Barber, K.R., Lordo, C., and Grant, C.W.M. (1988), Biochim. Biophys. Acta 939, 151-159

Melchior, D.L. and Steim, J.M. (1979) Prog. Surf. Memb Sci 14, 211-296.

Metcalf, T.N., Wang, J.L. and Schindler, M. (1986) Proc. Natl. Acad Sci. U.S.A. 83, 95-99.

Momoi, T., Ando, S. and Magai, Y (1976) Biochim. Biophys. Acta 441, 488-497.

Mullin, B.R., Fishman, P.H., Lee, G., Tedley, E.D., Winand, R.J., Kohn, L.D. and Brady, R.O. (1976) Proc. Natl. Acad. Sci. U.S.A 73, 842-846.

Mullin, B.R., Aloj, S., Fishman, P.H., Lee, F., Kohn, L.D. and Brady R.O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1697-1683

Nichols, J.W. and Pagano, R.E. (1982) Biochemistry 21, 1720-1726

Nichols, J.W and Pagano R.E. (1981) Biochemistry 20, 2783-2789.

Nichols, J.W. (1985) Biochemistry 24, 6390-6398.

Nicholson, G.L. (1976) Biochim. Biophys. Acta, 457, 57.

Nordlund, J.E., Schmidt, C.F., Dicken, S.N. and Thompson, T.E. (1981) Biochemistry 20, 3237-3242.

O'Brien J.S. and Rouser, G. (1964) J. Lipid Res. 7, 339-342.

Ohki, K., Yamauchi, T., Bunno, Y. and Nozawa, Y (1981) Biochem. Biophys. Res. Comm. 100, 321-327.

Ohnishi, S.-I and Ito, T. (1974) Biochemistry 13, 881-887.

Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727-2740.

Pagano, R.E., Martin, O., Schroit, A.J. and Struck, D.K. (1981) Biochemistry 20, 4920-4927.

Papahadjopoulos, D. and Weiss, I. (1969) Biochim. Biophys. Acta 183, 417-426

Papahadjopoulos, D., Poste, G., Schaeffer, B.E., Vail, W.J (1974) Biochim. Biophys Acta 352, 10-28.

Pschet, I and Sundell, S. (1977) Chem. Phys. Lipids 20, 175-191.

Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Biochim.Biophys. Acta 35-44.

Prestr, E.E. and Chan, S.I. (1982) Biochemistry 21, 3821-3830.

Radin, N.S. (1974) Lipids 9, 358-360.

Radin, N.S. (1976) J. Lipid Res. 17, 290-293.

Rechktenwald, D.J. and McConnell, H.M. (1981) Biochemistry 20, 4505-4510.

Reed, R.A., Mattai, J. and Shipley, G.G. (1987) Biochemistry 26, 824-832.

Reed, R.A. and Shipley G.G., (1987) Biochim. Biophys. Acta 896, 153-164.

Revesz, T. and Greaves, M. (1975) Nature 257, 103-106.

Rintoul, D.A., and Welti, R. (1989) Biochemistry 28, 26-31

Rintoul, D.A., Welti, R. and Song, W. (1988) Biophys J. 53, 1261.

Roseman, M.A. and Thompson, T.E. (1980) Biochemistry 19, 439-444.

Rouser, G., Nelson, G.J., Fleischer, S. and Simon, G. (1968) in Biological Membranes: Structure and Function (D. Chapman, ed., Academic Press, New York), pp.5-70.

Rujanavech, C. and Silbert, D.F. (1986) J. Biol. Chem. 261, 7215-7219.

Ruocco, M.J., Atkinson, D., Small, D.M., Skarjune, R.P., Oldfield, E., and Shipley, G.G. (1981) Biochemistry 20, 5957-5966

Ruocco, M.J. and Shipley, G.G. (1981) Biophys. J. 43, 91-101.

Ruocco, M.J. and Shipley, G.G. (1984) Biophys. J. 46, 695-707.

Ruocco, M.J. and Shipley, G.G. (1986) Biochim. Biophys. Acta 859, 246-256

Ruocco, M.J., Shipley, G.G. and Oldfield, E. (1983) Biophys J. 43, 91-101

Saffman, P.G., and Delbruck, M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3111-3113.

Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209-237.

Schmidt, C.F., Barenholz, Y., Huang, C. and Thompson, T.E. (1978) Nature 271, 775-777

Sedlacek, H.H., Stark, J., Seiler, F.R., Ziegler, W. and Wiegandt, H. (1976) FEBS Letters 61, 272-276.

Serratlich, F.M., de Haas, G.H., and Shipley, G.G. (1984), Biochemistry 23, 713-720.

Shimshick, E.J. and McConnell, H.M. (1973) Biochemistry 12, 2351-2360.

Siegel, D. (1984) Biophys J. 45, 399-420.

Silvius, J.R., Leventis, R., Brown, P.M. and Zuckermann, M. (1987) Biochemistry 26, 4279-4287

Silvius, J.R. (1986) Biochim. Biophys. Acta 857, 217-228.

Silvius, J.R. (1982) in Lipid-Protein Interactions (Jost, P. and Griffith, O.H., eds.), Vol 2 pp. 238-281, John Wiley and Sons, New York

Silvius, J.R. and Gagné, J. (1984a) Biochemistry 23, 3232-3240.

Silvius, J.R. and Gagne, J. (1984b) Biochemistry 23, 3241-3249.

Simon, S.A. and McIntosh, T.J. (1984) Biochim. Biophys. Acta 773, 169-172.

Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720-731.

Skarjune, R. and Oldfield, E. (1982) Biochemistry 21, 3154

Skarjune, R. and Oldfield, E. (1979) Biochim. Biophys. Acta 556, 208-218.

Somerharju, P.J., Virtanen, J.A., Eklund, K.K., Vainio, P., and Kinnunen, P.K.J. (1985) Biochemistry 24, 2773-2781.

Spiegel, S., Blumenthal, R., Fishman, P.H. and Handler, J.S. (1985) Biochim. Biophys. Acta 821, 310-318

Stamatatos, L., Leventis, R., Zuckermann, M.J., and Silvius, J.R. (1988) Biochemistry 27, 3917-3925.

Steck, T.L., Fredy, F.J., and Lange, Y. (1988) J. Biol. Chem. 263, 13023-13031.

Stewart, T.P., Hui, S.W., Portis, A.R. Jr. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 556, 1-9

Stewart, T.P., Hui, S.W., Portis, A.R., Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 556, 1-16.

Stockton, G.W. and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251-263.

Stumpel, J., Wacksch, A., and Tibl, H. (1981) Biochemistry 20 662-667

Tardieu, A., Luzzati, V. and Reman, F.D. (1973) J. Mol. Biol. 75, 711-733

Tate, M.W. and Gruner, S.M. (1987) Biochemistry 26, 231-236.

Tenchov, B.G. and Koynova, R.D. (1985) Biochim. Biophys. Acta 817, 380-391.

Thompson, T.E. and Tillack, T.W. (1985) Ann. Rev. Biophys. Chem. 14, 361-386

Tilcock, C.P.S., Bally, M.B., Farren, F. and Cullis, P.C. (1982) Biochemistry 21, 4596-4601.

Tillack, C.P.S., and Cullis (1981) Biochim. Biophys. Acta 641 139-201

Tillack, T.W., Wong, M., Viretta, M. and Thompson, T.E., (1982) Biochim Biophys. Acta 691 261-273

Tokutomi, S., Lew, R., Ohnishi S.-i. (1981) Biochim Biophys. Acta 643 276-282.

Untract, S.B. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4449-4457

Van Meer, G. and Simons, K. (1986) EMBO J., 5 1455-1464.

Van Dijck, P.W.M., Kafer, A.J., Oonk, H.O.J. and de Gier, J. (1977) Biochim Biophys. Acta 470, 58-69.

Van Dijck, P.W.M., de Kruijff, B., Verkleij, A.J., van Deenen, L.L.M., and de Gier, J. (1978) Biochim Biophys. Acta 512, 84.

van Dijck, P.W.M., de Kruijff, B., van Deenen, L.L.M., de Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576-587.

van Dijck, P.W.M. (1979) Biochim .Biophys. Acta. 555, 89-101.

van Deenen, L.L.M., and de Gier, J. (1974) "Lipids of the red cell membrane" in The Red Blood Cell (Surgenor, D ed), pp.147-213, Academic Press, New York.

Vance D.E. and Vance J.E. (1985) Chapter 2 of Biochemistry of Lipids and Membranes (Menlo Park, The Benjamin/Cummings Publishing Company) p.31.

Vasilenko, I., de Kruijff, B. and Verkleij, A.J. (1982) Biochim Biophys. Acta 685, 144-152.

Verkleij, A.J., de Maagd, R., Leunissen-Bijvelt, J., and de Kruijff, B. (1982) Biochim. Biophys. Acta 684, 255

von Dreele, P.H (1978) Biochemistry 17, 3993-3994

Wattenberg, B.W and Silbert, D.F. (198) J Biol Chem 258, 2284-2289.

White, D.A (1973) Biochim. Biophys. Acta Libr 3 (2nd Edition), 441-482.

Wilschut, J , Duzgunes, N , Fraley, R Papahadjopoulos, D (1980) Biochemistry 19, 6011.

Wu, S.H. and McConnell, H.M. (1975) Biochemistry 14, 847-854

Xu, H., Stephenson, F.A., and Huang, C (1987) Biochemistry 26, 5448-5453

Xu, H. and Huang, C. (1987) Biochemistry 26, 1036-1043.

Yeagle, P.L. and Young, J.E. (1986) J. Biol. Chem. 261, 8175-8181.

Yeagle, P.L., (1985) Biochim Biophys. Acta 817, 267-287.

Yechiel, E. and Edidin, M. (1987) J. Cell Biol. 105, 755-760.

Yu, R.K. and Ando, S. (1980) in Structure and function of Gangliosides (Svennerholm, L., Mandel, P., Dreyfus H. and Urban P.F., eds.), pp. 33-45, Plenum Press, New York.

Zwall, R.F.A. (1978) Biochim. Biophys. Acta 515, 163-207.