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EXAMINATION OF THE ROLE OF MEMBRANE DYNAMICS AND ORDER IN POTENTIATING THE PHYSIOLOGICAL EFFECTS OF LIPOPOLYSACCHARIDES

By

Bernard Dale Hummel

A DISSERTATION

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ABSTRACT

EXAMINATION OF THE ROLE OF MEMBRANE DYNAMICS AND ORDER IN POTENTIATING THE PHYSIOLOGICAL EFFECTS OF LIPOPOLYSACCHARIDES

By

Bernard Dale Hummel

Lipopolysaccharides (LPS) are complex glycolipids found on the surface of Gram-negative bacteria and are capable of eliciting a wide array of biological responses in animals. These responses are primarily due to the interaction of LPS with cells of the immune system and are responsible for the deleterious effects of Gram-negative septicemia. The studies presented here investigate the molecular mechanisms, particularly at the membrane level, by which LPS activates immune cells.

A new understanding of the structural features of LPS required to elicit a biological response has been obtained. Analysis of rhizobial LPS showed that they have a drastically different lipid structure from enterobacterial LPS. These differences were in the headgroup and fatty acyl structures as well as its charge functionalities. The biological activities of rhizobial LPS were also distinct. They gave a strong response in the *Limulus* amebocyte lysate and B-lymphocyte mitogen assays but only elicited a weak response in the mouse toxicity, pyrogen and tumor necrosis factor production assays. These results indicate that some physicochemical property of the lipid A portion of LPS, and not the individual structural functionalities *per se*, is critical for its biological activity.

The effects of LPS on the properties of membranes and its role in the activation of immune cells has been investigated. I demonstrate that enterobacterial LPS has a dramatic effect on the order and dynamics of membranes. My results show that LPS

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induces lateral separation of model membrane lipids into distinct domains and reduces the acyl chain mobility of the membrane. I also demonstrate that there is not a linear relationship between membrane acyl chain mobility changes induced by LPS and its biological activity.

Synthetic lipids that were designed to interact with lipid A were used to probe its mechanism of action. The synthetic lipids were effective inhibitors of the biological activities of LPS. The membrane dynamics and order changes induced by the synthetic lipid inhibitors were also investigated. My results show that the inhibitors increase the amount of fluid lipid species present in the membranes and disrupt the rigid LPS/lipid aggregates in the membrane. These lipid inhibitors demonstrate that the membrane perturbations induced by LPS are critical to its mechanism of action and that molecules that can offset this perturbation can inhibit its ability to elicit a physiological response.

To my parents, Don and Joan Hummel

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Finally

They stuck by

Without their

possible.

Finally, I would like to thank my wife, Jean, and two sons, Zachary and Adam. They stuck by my side at all times and put up with my unusual hours for many years. Without their sacrifices, support, and unconditional love, this dissertation would not be possible.

Thank you all

Ben

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CPM

CSF

DMEM

D-PBS

DPH-PC

DPPC

DSC

E. coli

EI

ELISA

EU

FRAP

GC

GC-MS

IFN

IL

KDO

L_α

$L_{\beta'}$

LAL

LBP

List of Abbreviations

CPM	counts per minute
CSF	colony stimulating factor
DMEM	Dulbecco's modified eagles medium
D-PBS	Dulbecco's phosphate buffered saline
DPH-PC	2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSC	differential scanning calorimetry
E. coli	*Escherichia coli*
EI	endotoxin inhibitor
ELISA	enzyme linked immunosorbent assay
EU	endotoxin unit
FRAP	fluorescence recovery after photobleaching
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
IFN	interferon
IL	interleukin
KDO	3-deoxy-D-*manno*-octulosonic acid
L_α	lamellar liquid crystalline phase
$L_{\beta'}$	lamellar gel phase
LAL	*Limulus* amebocyte lysate
LBP	lipopolysaccharide binding protein

LPS

LUV

NMR

P_3

R. meliloti

R. trifolii

S. typhimurium

TI

TNF

TPnA

LPS	lipopolysaccharides
LUV	large unilamellar vesicles
NMR	nuclear magnetic resonance
$P_{\beta'}$	ripple phase
R. meliloti	*Rhizobium meliloti*
R. trifolii	*Rhizobium trifolii*
S. typhimurium	*Salmonella typhimurium*
T1	spin-lattice relaxation time
TNF	tumor necrosis factor
TPnA	*trans*-parinaric acid

Chapter 1

Introduction to Biological Membranes and Lipopolysaccharides

Introdu

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Introduction

Biological membranes are one of the critical components of cells. They function, in part, to compartmentalize cells and allow the processes of the cell to be conducted in a controlled environment. The isolation of prebiotic reactions may have been an essential step in the evolution of life itself. Thus it is likely that membranes were features of the earliest forms of life on earth (1).

Membranes have a common structural feature; they are all composed of amphiphilic molecules (having both hydrophobic and hydrophilic moieties) called lipids. It is energetically unfavorable for the hydrophobic regions of the lipids to interact with water. In the presence of water, lipids aggregate in such a way as to sequester their hydrophobic regions away from water. The unfavorable interaction of the hydrophobic regions of lipids with water is the fundamental driving force for membrane formation and is called the hydrophobic effect (2). Due to the hydrophobic effect, lipids are able to self aggregate (without the aid of other molecules or covalent bonds) into highly defined structures.

As early as 1925, it was postulated that membranes were composed of a bilayer of lipids (3). This structure allows the hydrophobic regions of the lipids to be sequestered towards the center of the bilayer and away from water. The hydrophilic regions are on the surface of the membranes and are free to interact with water. There are many different three-dimensional conformations, or phases, that the bilayer can form. These will be discussed in more detail later in this chapter. However, it is the sheet-like, lamellar structure that encapsulates cells and is, therefore, central to the studies of biological membranes.

In 1972, Singer and Nicolson proposed the fluid mosaic model to explain the structure, organization and motion of biological membranes (4). This model proposed that biological membranes are a bilayer of lipid molecules that serve as a matrix for membrane associated proteins (Figure 1.1). The lipids and proteins are free to diffuse in the plane of the membrane. Thus, the membrane is viewed as a two dimensional viscous fluid in which the lipids and proteins are randomly distributed. Although we now know that this model is much to simple to explain the structure and function of biological membranes, it has served as the foundation upon which all the modern membrane research has been based.

Biological membranes are extremely complex systems. This is highlighted by the number of different molecular species that may be present in a membrane. There are many different classes of lipids (>10) which may be present (a lipid class is defined by its headgroup and backbone structures). These lipids typically have two hydrocarbon chains attached, of which there are over 20 different possibilities. On top of this lipid heterogeneity, there are potentially hundreds of different proteins associated with the membrane. This diverse set of molecules is part of an aggregate whose properties depend on their intermolecular interactions and motions.

This chapter on biological membranes can only begin to scratch the surface of the enormously diverse areas of research in membrane biology that has filled the pages of countless journal articles and books. I begin this chapter with an overview of the structure of lipids and the physical foundation of their organization. Next I discuss the motion (dynamics) and order (structure and organization) of lipids and the role they have in the function of the membrane. I end this chapter by discussing a class of lipids known

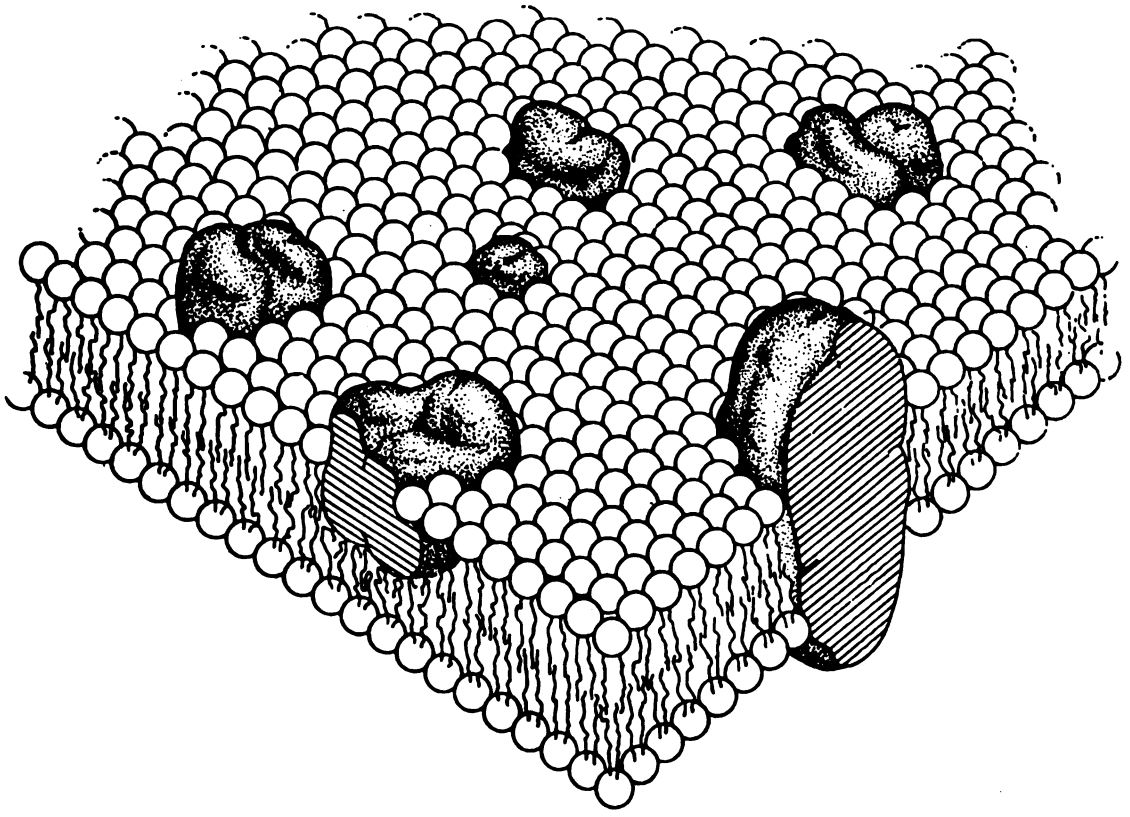


Figure 1.1: The fluid mosaic model of biological membranes in which the proteins and lipids form a two-dimensional viscous fluid. The proteins and lipids are free to move randomly in the plane of the membrane. Adapted from reference 4.

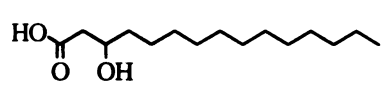
as lipopolysaccharides. These are unique and special lipids that are capable of eliciting a very swift and severe immune response. It is the mechanism of their action that is at the heart of this thesis.

Structure and Phase Behavior of Membrane Lipids

A tremendous diversity of lipid structures are found in biological membranes including fatty acids, glycerophospholipids, sphingolipids, glycolipids, steroids, and some vitamins. The structures of some of these lipids are shown in Figure 1.2. A more complete review of membrane lipid structures can be found in reference 5. All of these lipids have a common structural motif. They all have a non-polar, hydrophobic region and a polar, hydrophilic region. The important differences lie in the relative proportions of these two regions present, the charge state of the molecule, and the three dimensional conformation which it adopts.

The class of lipids known as glycerophospholipids comprises the bulk of the membrane lipids in eukaryotes. Their basic structural unit is a phosphoglycerol. To the phosphate is attached one of several different polar molecules which generates the hydrophilic headgroup of the lipid. Fatty acids are attached to the hydroxyl groups of the glycerol to provide the hydrophobic region of the lipid. An example of a glycerophospholipid is dipalmitoylphosphatidylcholine (see Figure 1.2). This lipid has a choline group attached to the phosphate and two 16-carbon saturated fatty acids (palmitic acids) attached to the glycerol.

The phase (aggregate structure) that a given set of lipids will form depends on the intra- and inter-molecular forces of the lipid molecules. The hydrocarbon chain region of



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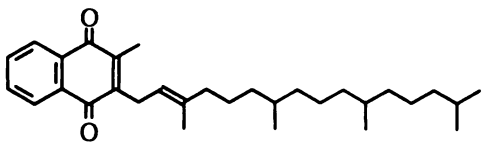

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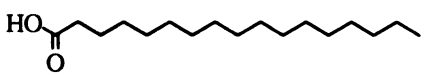

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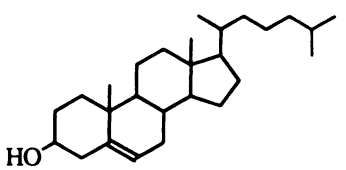

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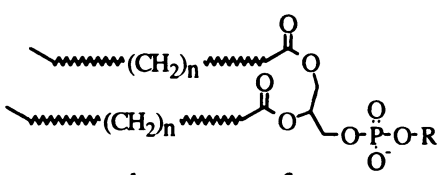

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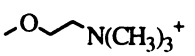

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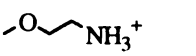
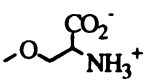


phosphatidylethanolamine



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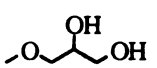

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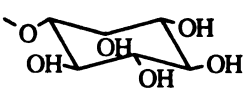

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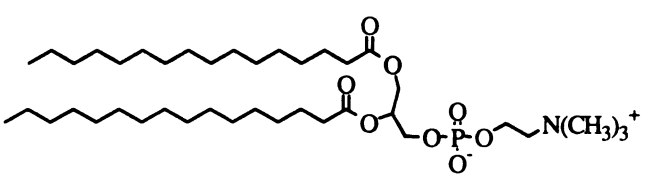

dipalmitoylphosphatidylcholine

Figure 1.2: Some common membrane lipids.

the lipid experiences steric repulsive and van der Waals attractive forces. The polar headgroup of lipids are influenced by steric repulsion, electrostatic interactions and hydration forces (6). The molecular forces of both the hydrophobic and the hydrophilic regions of the lipids are balanced to provide the minimum free energy of the lipid aggregate. Thus, this delicate balance of forces dictates their aggregate properties and, hence, their phase behavior.

The balance of molecular forces in the lipid aggregate results in each of the lipid molecules having an effective (average) geometry. The packing constraints of this geometry determine the overall 3-dimensional shape that the membrane will adopt (6). This is illustrated in Figure 1.3. For instance, a lipid that has a cylindrical shape will tend to pack together in planar sheets, thus giving rise to a lamellar phase. If, however, the headgroups experience charge repulsion, their geometry may change and result in the lipids adopting a different phase.

It can easily be seen that any factor that alters the delicate balance of intramolecular and intermolecular forces can alter the phase state of the lipids. These factors include temperature, pressure, hydration, pH, or ion concentration. The addition of exogenous lipophilic molecules (such as proteins, antibiotics, organic solvents, or lipids) will also perturb this balance of forces. If this perturbation causes the lipids to favor a different aggregate structure, they will undergo a phase transition to the new, lower energy phase. For example, below 35°C dipalmitoylphosphatidylcholine (DPPC) is in the lamellar gel ($L_{\beta'}$) phase (Figure 1.4). When this lipid is heated to 35°C, it undergoes a transition to the ripple ($P_{\beta'}$) phase. Continued heating results in another phase transition at 42°C. The thermal energy imparted into the system causes the lipids

Figure 1.3: Relationship between molecular geometry of lipids and the phases they adopt. Taken from reference 7.

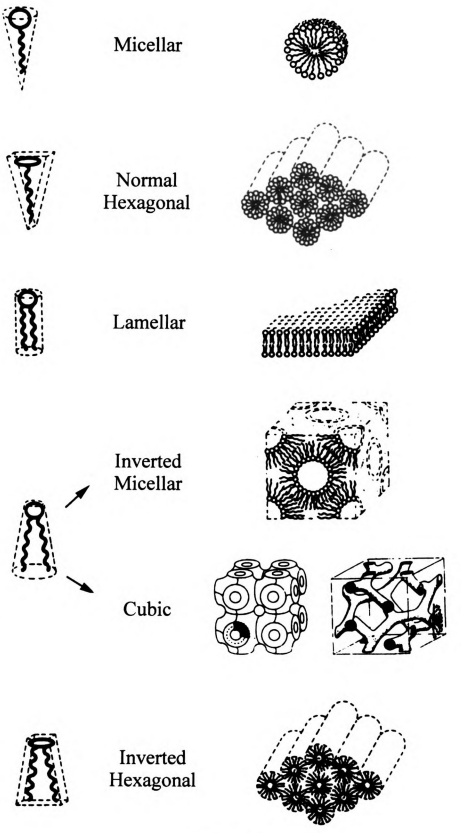
Micellar
Normal
Hexagonal
Lamellar
Inverted
Micellar
Cubic
Inverted
Hexagonal

Figure 1.3

to undergo *trans-gauche* isomerizations around the carbon-carbon bonds of the acyl chains and thus increase their motion. This is accompanied by the increased hydration of the lipid headgroup and an increase in the overall area occupied by each lipid molecule. The result is the lamellar liquid crystalline (L_α) phase. This is one example of lipid polymorphism. Of course, the types of phases that a membrane will adopt and the temperature (or other factors) which will cause a phase transition will be different for each type of lipid studied.

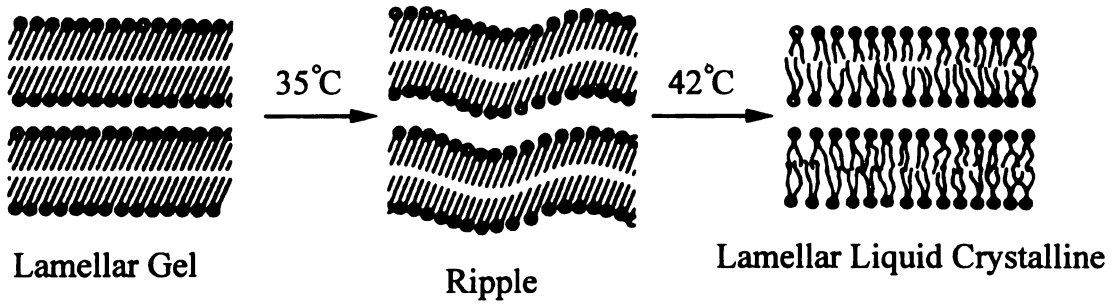

Figure 1.4: A schematic of the phase behavior of dipalmitoylphosphatidylcholine as a function of temperature. Modified from reference 8.

Membrane Dynamics

The fluid mosaic model of biological membranes highlights one of the most fundamental properties of membranes: their motion. Membranes are ordered two-dimensional fluids that exhibit a range of different types and rates of motions while maintaining a large degree of order. Therefore, they have the properties of both fluids and crystals. Hence the general term, liquid crystals, is used to describe materials which

can form this unique state of matter. Membrane dynamics are one of the critical factors that determine the physical state of the membrane. As will be discussed later in this chapter, the dynamic state of a membrane also influences its function.

There are several types of motions (dynamics) that occur in membrane lipids. These motions range from the very fast vibrations of the C-H bonds to the very slow translation of lipids from one side of the bilayer to the other. Concomitantly, there are a range of techniques which are sensitive to different time scales of motion and are used to study membrane dynamics. The types and rates of motions found in membranes and the time scales of the techniques used to study them are summarized in Figure 1.5. Membrane dynamics are often described in terms of its 'fluidity'. Indeed, the membrane literature in inundated with this term. Fluidity is simply the inverse of viscosity and viscosity is defined as the amount of resistance to motion in a medium. Fluidity and viscosity are only valid when applied to isotropic substances and, therefore, are not rigorously correct terms to describe anisotropic systems such as membranes. When used to describe membranes, fluidity and viscosity are used in a more qualitative sense. In reality, they describe a combination of motions. Therefore, they preclude the possibility of providing a detailed picture of the molecular motions being measured and their use should be avoided when such information is desired.

Lipid hydrocarbon chain motions constitute some of the fastest modes of motions found in membranes and are also one of the most critical when trying to define its dynamic state. They result from the *trans-gauche* isomerizations of the carbon-carbon bonds in the acyl chains. Lipids that are in a rigid, solid-like phase (such as the $L_{\beta'}$ phase of DPPC) have nearly all of their carbon-carbon bonds in an all-*trans* configuration. In this configuration, the acyl chains are straight and can pack tightly together. Lipids that

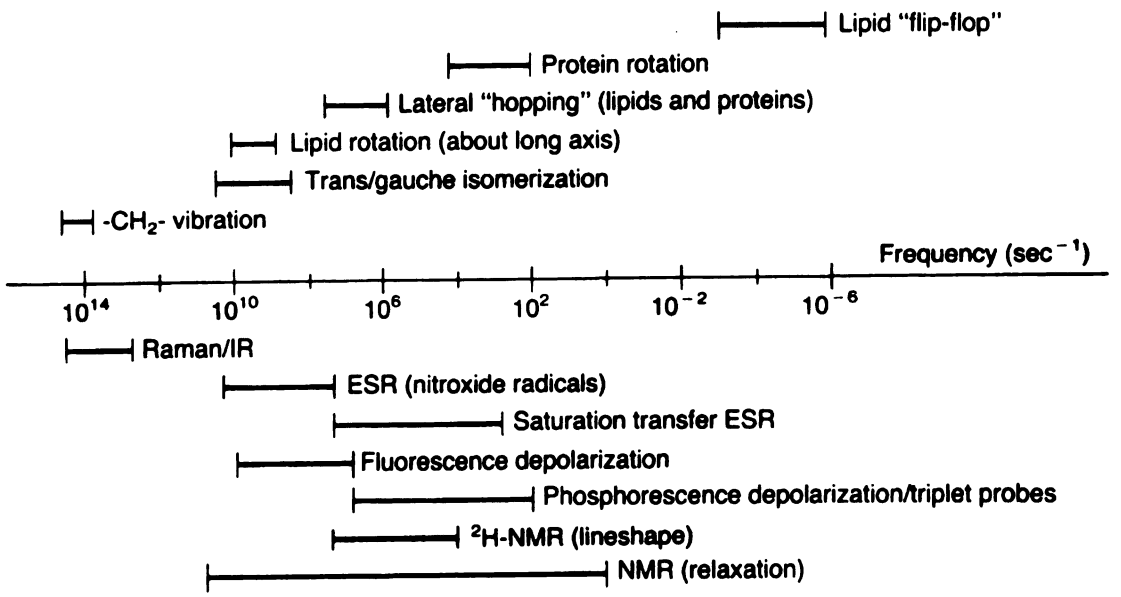

Figure 1.5: Rates of membrane motions and the techniques that are sensitive to these motions. Taken from reference 9.

are in a fluid-like phase (such as the L_α phase in DPPC) have an increased number of *gauche* conformations in their acyl chains. The *gauche* conformations put 'kinks' in the acyl chains and results in their inability to pack tightly together. Using infrared spectroscopy, Casal and McElhaney have demonstrated that there is an average of 3.7 *gauche* conformers per acyl chain of DPPC at 5°C above the gel ($P_{\beta'}$) to lamellar-liquid crystalline phase transition (10). The *trans-gauche* isomerizations of the carbon-carbon bonds are constantly changing their position along the acyl chain.

A more detailed analysis of the lipid acyl chain motion has been performed by Peterson and Chan (11). They have postulated that there are two major modes of motions in the acyl chains of lipids. The first type of motion arises from large fluctuations of the acyl chain as the result of single *gauche* conformers. A single *gauche* conformer would produce a bend in the acyl chain and produce a large structural perturbation, especially if it is located near the lipid headgroup. This large structural perturbation would be unfavorable due to the steric interactions with neighboring lipids. Thus, this type of motion is expected to be slow. Peterson and Chan (11) have suggested that this type of acyl chain motion occurs at a rate of approximately 10^{-8} s. The unfavorable large perturbation induced by a single *gauche* conformer can be partially overcome by a second *gauche* conformer next to the first but in the opposite direction. Thus the acyl chain will have neighboring *gauche(+)* and *gauche(-)* conformers resulting in a kink. A kink in the acyl chain results in a much smaller structural perturbation than a single *gauche* conformer and is therefore less energetically expensive. By successive *trans-gauche(+)/gauche(-)-trans* isomerizations, the kink can translate up and down the acyl chain. This is the second mode of acyl chain motion and is called 'kink diffusion'. The rate of *trans-gauche* isomerizations that results in kink diffusion is approximately 10^{-10} s.

Membrane Order

The hydrophobic effect causes lipids and proteins to self-assemble into large ordered aggregates (membranes). Order, therefore, is an inherent property of any membrane. There are three basic types of membrane order: orientational order, asymmetry, and lateral order. Orientational order is the degree to which the membrane molecules adopt the same orientation relative to the bilayer normal. Asymmetry refers to the amount of similarity between the two halves of the bilayer. The final type of order is a measure of the lateral organization of the molecules in the plane of the membrane. I will focus this discussion on this latter type of membrane order. An excellent discussion of orientational order and asymmetry can be found in reference 12.

The fluid mosaic model proposed that the molecules of biological membranes are, for the most part, free to move randomly in the plane of the membrane (4). Moreover, this motional freedom results in an essentially homogeneous lateral distribution of molecules in the membrane. We know now that this is not true. Membrane components readily laterally separate into regions (domains) of distinct compositions, physical properties and motions. Membrane domains usually refer to a lateral segregation of lipids. They may or may not be accompanied by the lateral segregation of proteins.

The balance of entropy and intermolecular interaction energy (6) decides whether or not lipids form domains. If a lipid has a particular structure that results in the strong interaction with a neighboring lipid, then the two molecules will tend to remain together. If this interaction is weak, however, then the entropy of mixing predominates and the lipids will diffuse apart. This delicate balance of interaction energy and entropy results in a dynamic equilibrium in which those lipids that have a stronger energy of interaction will tend to associate together into distinct domains. Any perturbation in this equilibrium,

such as changes in temperature, pressure, pH, or the addition of lipophilic molecules, will result in a change in the domain organization of the membrane. Depending on its composition, each domain will have different structural, physicochemical and motional properties.

The most definitive and elegant studies on membrane domains have been performed with lipid monolayers (see ref. 13). In these experiments, synthetic lipids are doped with fluorescent lipid analogs and allowed to assemble at the air-water interface. The lipid monolayers are directly visualized by epifluorescence microscopy as a function of surface pressure, temperature, or other factors. Peters and Beck (14) were the first to visualize the formation of domains by this method during the 'fluid'-to-'crystalline' phase transition. Studies by McConnell and co-workers as well as Miller and Möhwald have greatly expanded these initial findings. They found that lipids, even those of a pure molecular species, exhibit regular, periodic arrays of domains whose shape can be highly complex and variable (15-17). These domains not only had distinctive shapes, but they also had characteristic motional (15) and physical properties including their electrostatic dipole moment (18, 19). Furthermore, these studies also demonstrated that the size and shape of lipid domains are extremely sensitive to small amounts of added cholesterol (20, 21). Thus it is obvious that even small changes in lipid composition can lead to dramatic long-range perturbations in membrane order.

Given the ability of membranes to form domains even with the very pure lipids systems used in the monolayer studies, it is perfectly reasonable to expect that more complex lipid systems will also be able to laterally segregate into domains. This is, in fact, true for both biological membranes and liposomes composed of various lipid mixtures. In the latter case, mixtures of lipids have been shown to exist in domains by

using differential scanning calorimetry (22, 23) and by measuring the steady state and time resolved emissions from fluorescent probes (24-26). More direct evidence of domain formation in phospholipid vesicles has been provided by Haverstick and Glaser (27). This study visualized the formation of acidic phospholipid rich domains induced by Ca^{2+} using epifluorescent microscopy and fluorescently labeled lipids.

Lipid domains have also been observed in cellular membranes. Some cells have large, usually morphologically distinct, regions of their membranes that contain specific lipid and protein compositions. This type of 'macrodomain' has been observed in polarized epithelial cells (28), the sperm cell plasma membranes (29), inner- and outer-membrane adhesion zones in gram-negative bacteria (30), and the thylakoid membranes of chloroplasts (31).

Cellular membranes also have smaller membrane domains, sometimes referred to as 'microdomains'. Due to the inherent structural complexity of cellular membranes, we should expect that membrane microdomains are ubiquitous. However, because these domains are relatively small and can not be isolated, studies on them have been difficult and slow to evolve. We are now just beginning to understand and measure the properties of these membrane domains. Lipids may be reorganized by their interaction with membrane associated proteins. This has been observed for gramicidin and D-β-hydroxybutyrate dehydrogenase (32), pyrularia thionin (33), and some ATPases (34, 35). Of course, domains may also be created by the lateral segregation of the lipids themselves.

A wide variety of methods have been employed to detect domains in biological membranes (see ref. 36). Several of these studies provide definitive evidence for their existence. An innovative use of fluorescence recovery after photobleaching (FRAP) has

been used by Yechiel and Edidin to detect domains in the plasma membrane of fibroblasts (37). By varying the diameter of the laser beam, they were able to demonstrate a large lateral heterogeneity in the diffusion rates of a fluorescent lipid. Yu et al. (38) were also able to demonstrate the existence of domains in fibroblasts cell membranes. These authors used a unique fluorescent probe, laurdan, and two-photon scanning microscopy to demonstrate that there is lateral heterogeneity in the plasma and nuclear membranes. The membrane of erythrocytes has also been shown to contain lipid domains by fluorescence microscopy (27, 39, 40).

The reports discussed above demonstrate that all membranes, even those composed of very pure lipid species, can laterally segregate into domains. These domains will have different physical and functional properties. We must now focus not only on detecting these domains, but also on developing an understanding of their physical properties, what affects their formation and, most importantly, how they affect the function of membranes.

So far, I have treated membrane dynamics and order as if they were separate entities. However, it is important to realize that the motions and organization of membranes are tightly coupled. If one type of membrane motion is altered, other modes of motion as well as its organization may also be altered. These membrane properties are intimately interconnected and can never be isolated. By measuring some of the modes of dynamics or order, we are attempting to understand the membrane as a whole. Clearly, the complexity of membranes makes this ultimate goal extremely difficult to achieve.

Physiological Relevance of Membrane Dynamics and Order

Traditionally, lipids have been viewed as the inert matrix in which membrane proteins are located. We now know that lipids play a vital role in the function of the membrane. It is clear that, on top of their structural role, lipids actively participate in signal transduction events and are the substrates for some enzymes. However, the dynamics and order of lipids also play an important role in regulating the functions of the membrane.

The degree of membrane order and motion (often described as 'fluidity') has long been thought to be critical to its function. Biological membranes generally have a significant proportion of their lipids in the fluid-like liquid-crystalline phase and it was proposed that normal membrane functions require this phase (41). The physiological relevance of membrane dynamics and order is highlighted by studies on the adaptations of organisms to environmental stress. Many organisms alter their membrane composition in response to environmental perturbations (such as temperature, pH, organic solvents, etc.) in order to maintain a constant dynamic state of the membrane (see ref. 42, 43). This phenomenon has been called homeoviscous adaptation (44). Clearly, the maintenance of a particular dynamic state of the membrane is critical to normal cellular functions.

The intimate interactions of membrane associated proteins with lipids suggest that the functions of these proteins may be modulated by the physical state of the membrane. A change in the 'fluidity' of the membrane has been correlated with a change in the activity of several enzymes including succinate cytochrome c reductase, phospholipase A_2, various ATPases, adenylate cyclase, acyl CoA cholesterol acyl transferase, glucose-6-phosphatase, and fatty acid desaturase (see ref. 45). Furthermore, many membrane-

associated enzymes display discontinuous Arrhenius plots, and the breaks in the Arrhenius plots are often associated with the motional state of the membrane (45).

While the above mentioned examples lend credence to the idea that the dynamic state of the membrane can regulate protein function, they do not examine the full scope of lipid-protein interactions. Many of these reports use exogenous lipids, such as cholesterol, to alter the motional state of the lipids. However, other physical properties of the membrane may also be altered in these studies. In fact, cholesterol is known to affect membrane properties in a very complex manner. It increases the order of fluid lipids (46) as well as dramatically affecting the size and shape of lipid domains (20). As discussed above, membrane dynamics and order are interconnected. The need to investigate other aspects of the physical properties of membranes is emphasized by studies that argue that 'fluidity' *per se* may not be the critical factor regulating the activity of membrane bound enzymes (47-49). These reports suggest that other physical properties of membranes may be involved. Indeed, it has been demonstrated that phospholipase A_2 (PLA_2) is most active at the onset of the bilayer-to-nonbilayer phase transition (50, 51). This indicates that PLA_2 activity is sensitive to the bilayer packing stress and defects. Senisterra and Epand (52) observe a similar activity dependence with protein kinase C (PKC). They observed that the activity of PKC is not simply regulated by membrane defects, but is also closely associated with the hexagonal phase propensity of the membrane. They were unable to determine the exact reason for this dependence. To this end, Tate et al. have proposed that enzyme activity can be modulated by the build up of elastic strain in the membrane as a result of non-lamellar forming lipids (53).

The lateral organization of lipids has also been demonstrated to be a critical determinant of membrane function. Indirect evidence for this is provided by studies on

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the effects of aging on myocyte and fibroblast cell membranes (54-57). These studies demonstrate that the lipid compositional changes seen in aging cells results in a change in the lateral mobility and organization of the membrane. These changes are reversed by the addition of the depleted lipid species. Furthermore, they demonstrated that these organizational changes which occur with aging resulted in a disruption of the muscarinic receptor activity and ability to couple to G-proteins. Additionally, Asturias et al. have shown that the structural and functional changes in the Ca^{2+}-ATPase of sarcoplasmic reticulum membranes is closely associated with the lateral organization of the lipids (58). More direct evidence for the role of lipid domains in the membrane function has been demonstrated with phospholipase A_2. This enzyme has been shown to target and hydrolyze solid-like lipid domains (59). Moreover, after a critical amount of the solid-phase domains have been hydrolyzed, the enzyme inactivates itself by self-aggregating into proteinacious domains in the membrane. In an elegant study by Dibble et al. (60), the activity of protein kinase C was found to be strongly dependent on the formation of membrane domains with specific lipid compositions. They also demonstrated that, under some conditions, the coexistence of domains with different lipid compositions was favorable for PKC activity.

Using model membranes and FRAP, Ameida et al. (61) have observed that the lateral diffusion of lipids exhibits large and sudden changes with changes in the lipid composition. They interpreted their results in terms of membrane domains and percolation theory. As the lipid composition (or other parameter such as temperature) changes, the solid-lipid domains (for example) grow as a result of an increase in the number of lipids in that phase. At a critical point, the solid-lipid domains that were previously isolated from one another now become connected. This is called the

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percolation threshold. At the percolation threshold, lipid domains that were previously disconnected become continuous and visa-versa. This results in a sudden and dramatic change in the lateral mobility of the lipids contained in these domains. Interestingly, Muderhwa and Brockman have demonstrated that pancreatic lipase activity is strongly dependent on lipid domain percolation (62). Because membrane proteins are preferentially soluble in specific lipid domains (63), the organization, size, composition and connectivity of membrane domains can provide a critical mechanism for the regulation of membrane function. This has been demonstrated in a theoretical analysis of membrane reactions in which it was found that domain size and connectivity can alter the rates and yields of enzymatic (or other) reactions in the membrane (64). Thus, the lateral segregation of membranes into domains provides a mechanism for cells to create specific functional areas within the membrane.

Since it is now well established that domains are found in biological membranes, it is obvious that these domains are likely to be critical to the normal function of cell membranes. Less clear is a molecular understanding of the physical and functional properties of the domains. In the discussions above, I have given some insights into how the physical properties of membranes can control the activity of enzymes. One can also see how domains can create functional areas in the membrane and can control the interactions of substrates and proteins in the membrane. The organization of the membrane can, however, have additional roles in its function. For example, it has been demonstrated that lipids can conduct protons laterally on the surface of the membrane (65). This results in the efficient coupling of spatially separated proton pumps. The protons move via an H-bond network that is critically dependent on the conformation of the lipid headgroups. Domains of differing lipid compositions and conformations would

define the boundaries of the proton travel and therefore affect the proton motive force. Membranes are also known to be ion storage devices (66). Ions, especially Ca^{2+}, are critical to normal cellular functions and signal transduction events. Interestingly, Ca^{2+} can affect domain formation (27). Conversely, disruption of these Ca^{2+} rich domains will release Ca^{2+}. Thus, membranes are capable of absorbing and releasing Ca^{2+} in a spatially defined way. Finally, membrane 'defects' found at the boundaries of domains may induce special functions. I discussed above that membrane defects might be associated with altered protein function (50-52). In addition to this, regions of membrane defects cause an increase in the permeability of the membrane to water and small molecules (67, 68).

From the above discussions, it is easily seen that membrane dynamics and order play a major role in the function of the membrane, which, in turn, can control the physiology of the cell. Therefore, there is an intimate and dynamic coupling between the physical state of the membrane, its function, and the function of the cell. This coupling is summarized in Figure 1.6. Understanding the interplay between the dynamics and order of the membrane and the physiology of the cell is essential to grasp an understanding of membrane biology. While we know that this connection exists, we have only begun to discover the full breadth of its potential.

In the sections above, I have discussed membrane structure, motions and organizations. Furthermore, I reviewed how these membrane properties might influence membrane and cellular function. In the remainder of this chapter, discussions will focus on an unusual class of lipids (lipopolysaccharides) with an amazing ability to alter the function of cells. The ability of lipopolysaccharides to alter cellular functions results in a dramatic affect on the immune system of animals. As will be seen in the remainder of

Figure 1.6: The coupling between membrane dynamics and order, the function of the membrane, and the physiology of the cell.

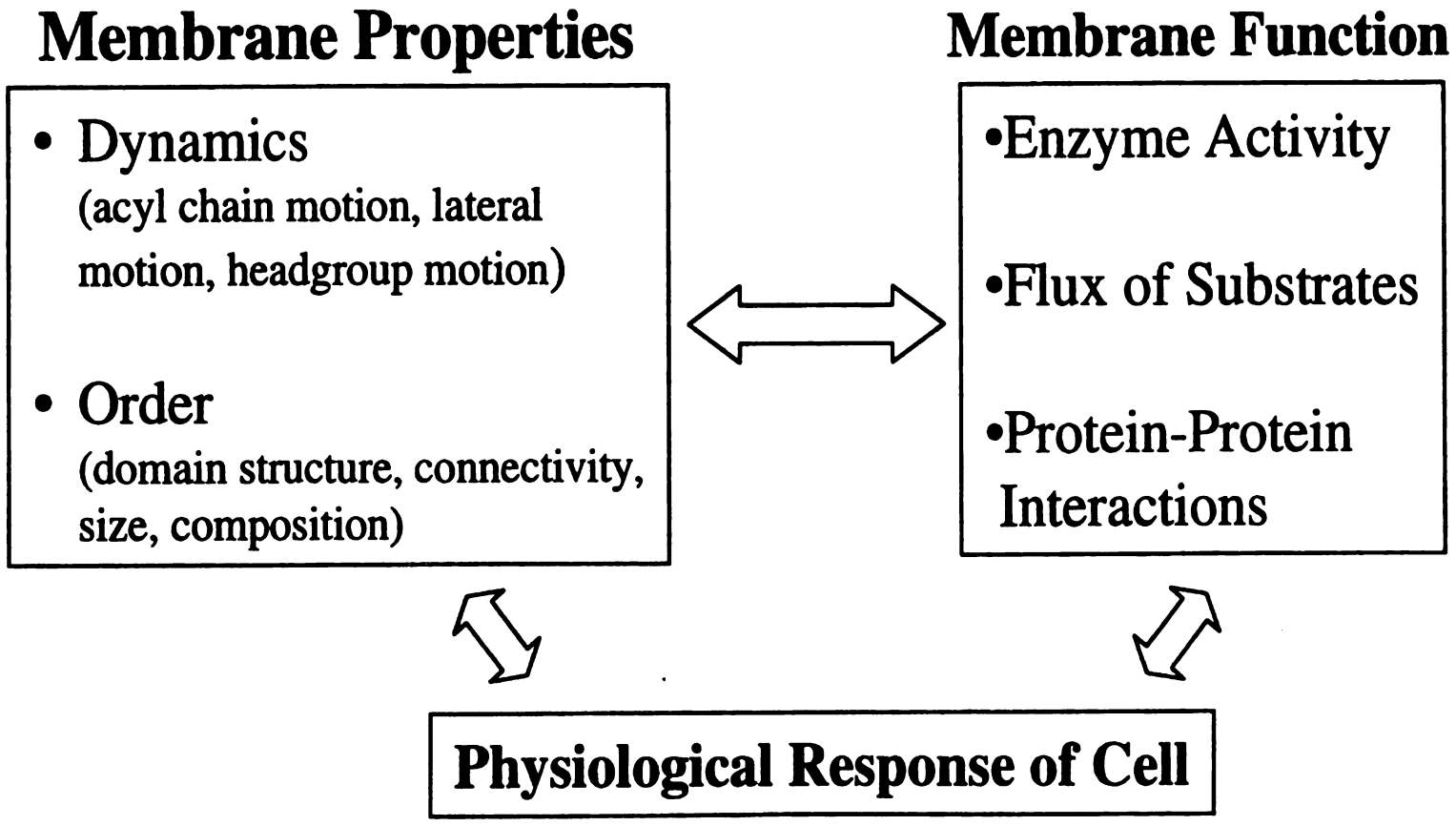
Membrane Properties
Membrane Function
• Dynamics
(acyl chain motion, lateral motion, headgroup motion)
• Order
(domain structure, connectivity, size, composition)
•Enzyme Activity
•Flux of Substrates
•Protein-Protein Interactions
Physiological Response of Cell

Figure 1.6

this dissertation, understanding how lipopolysaccharides affect membrane dynamics and order is important to understanding its mechanism of action.

Lipopolysaccharides

Lipopolysaccharides (LPS) are a unique class of glycolipids found on the surface of Gram-negative bacteria (Figure 1.7). LPS are capable of eliciting a wide array of biological responses when they interact with cellular systems of animals. A list of some of these biological responses is given in Table 1.1. Some of these responses are potentially beneficial, such as the ability to stimulate the immune system, to act as an adjuvant and to induce tumor necrosis. Other effects, such as the induction of fever, blood pressure depression, shock, and disseminated intravascular coagulation, can be harmful. In fact, in severe cases, the biological effects elicited by LPS lead to the death of the individual. A discussion of the mechanisms of these biological effects will be given later in this chapter.

Lipopolysaccharides were first discovered by Richard Pfeiffer in 1892. He found that Gram-negative bacteria produce toxins that are distinct from the more commonly known exotoxins in that they were heat-stable and remained associated with the bacteria. Due to these unique properties, he called them endotoxins (see ref. 69). Since this first discovery over 100 years ago, a tremendous amount of effort went in to elucidating the chemical structure of endotoxins. It was found that they consisted of both lipid and polysaccharides. Thus a name reflecting its chemical composition, lipopolysaccharides, was given. Today both endotoxins and lipopolysaccharides are used essentially interchangeably although the latter term usually refers to the more highly purified form.

The majority of structural work on lipopolysaccharides has been accomplished

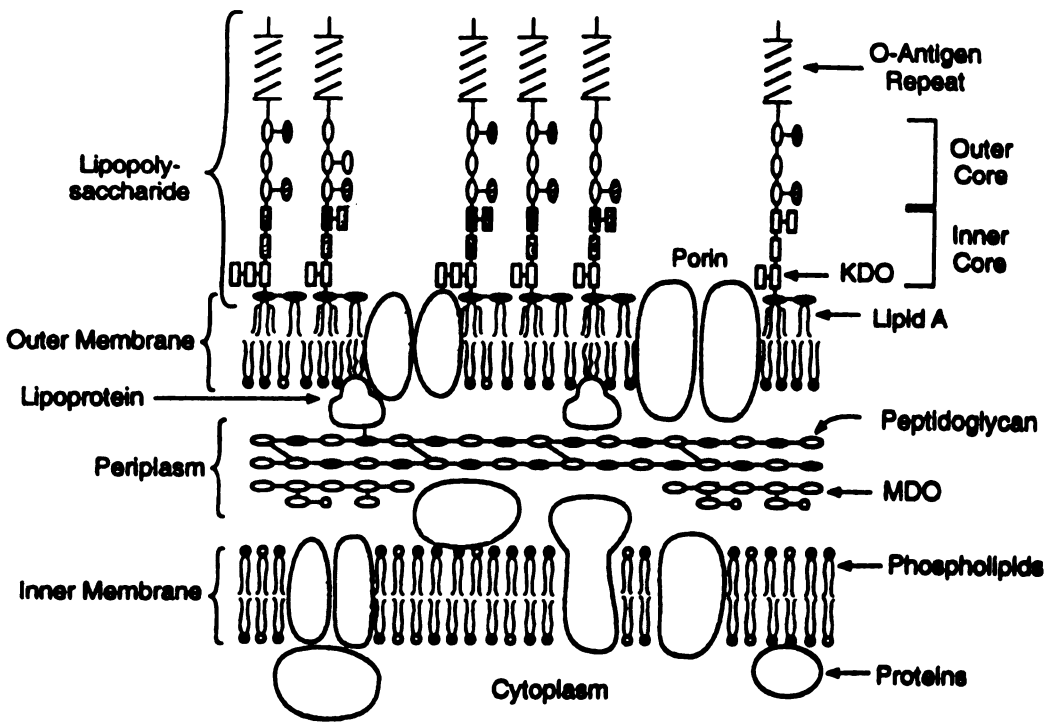

Figure 1.7: Outer envelope of Gram-negative bacteria. Lipopolysaccharides are located on the outer leaflet of the outer membrane.

with enteric bacteria such as *Escherichia coli* or *Salmonella*. Therefore, discussions of LPS structure imply that they are derived from these bacteria unless otherwise noted. LPS are composed of two regions, a lipid region and a polysaccharide region (Figure 1.8). The polysaccharide region is further divided into the O-specific polysaccharide and the core oligosaccharide. The O-specific polysaccharide (O-antigen) is composed of repeating oligosaccharide units (see ref. 70). An O-polysaccharide from wild type LPS will have between 0-40 oligosaccharide repeats. The core oligosaccharide is linked to the lipid moiety by an unusual sugar, 3-deoxy-D-*manno*-2-octulosonic acid (KDO). This eight-carbon sugar is acid labile and facilitates the chemical cleavage of the lipid and polysaccharide regions. The core oligosaccharide may also contain pentoses, hexoses, heptoses, amino sugars and some substituents such as phosphate and phosphorylethanolamine (see ref. 70, 71).

Table 1.1: Some typical biological activities elicited by lipopolysaccharides.

- Lethal toxicity
- Pyrogenicity
- Bone Marrow Necrosis
- Hypotension
- Platelet aggregation
- Complement Activation
- Adjuvant Activity
- Induction of Tumor Necrosis
- Activation of clotting enzyme cascade of the lysate of *Limulus* amebocytes
- Induction of prostaglandin synthesis
- Induction of tumor necrosis factor
- Induction of interferon
- Induction of interleukins
- Induction of colony stimulating factor
- Mitogenic for B-lymphocytes
- Macrophage activation
- Endothelial cell activation

The lipid region of LPS is called lipid A. The structure of lipid A, solved in 1983 (72), consists of a β-1,6 linked glucosamine disaccharide headgroup that is phosphorylated at the 1 (α) and 4' positions (Figure 1.9). Lipid A contains a total of six fatty acids. Four 3-hydroxyl tetradecanoic fatty acids are attached directly to the glucosamines by amide and ester linkages at the 2, 3, 2' and 3' positions. The 3-hydroxy groups of the 2' and 3' fatty acids are esterified by a dodecanoic and a tetradecanoic fatty acids, respectively. This structure was confirmed when it was produced by total organic synthesis and was found to be indistinguishable from bacterial-derived lipid A (73). Because this structure has the highest level of biological activity, it is considered to be the 'typical' or 'model' lipid A. However, some degree of heterogeneity is found in all lipid A preparations. Additionally, lipid A derived from bacteria other than *E. coli* or *Salmonella* may contain significantly different structures (see ref. 74).

Lipopolysaccharides (and lipid A) have not lent themselves easily to physicochemical studies. This is partially due to the intrinsic heterogeneity of LPS preparations. Further complications arise from differences in experimental conditions. As with phospholipids, the physical properties of LPS are sensitive to environmental conditions such as temperature, pressure, water content, pH, and ions. The negatively charged groups in LPS make them extremely sensitive to these latter two parameters (75, 76). Cations such as Ca^{2+} and Mg^{2+} are especially critical to the physical properties of LPS since they are capable of binding to the phosphates of lipid A and cross-bridging them together to form a tight complex. These factors have made even the simple observation of the chain melting transition produce tremendously variable results (7).

Figure 1.8: Schematic diagram of the structure of lipopolysaccharides.

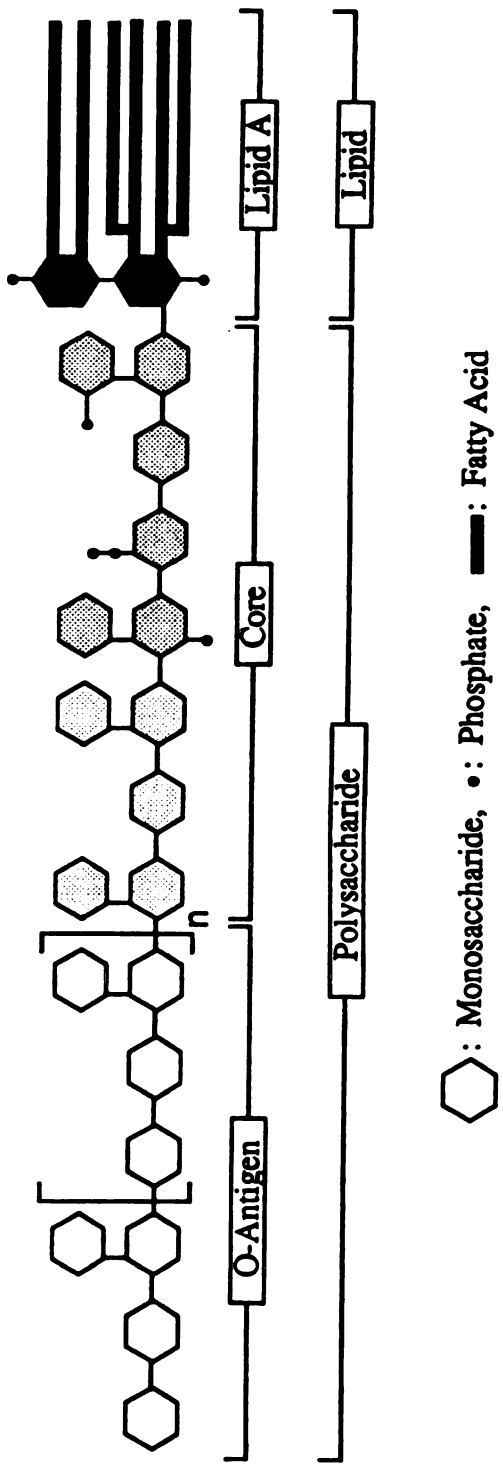
Lipid A
Core
O-Antigen
n
Lipid
Polysaccharide
: Monosaccharide, •: Phosphate, ▬: Fatty Acid

Figure 1.8

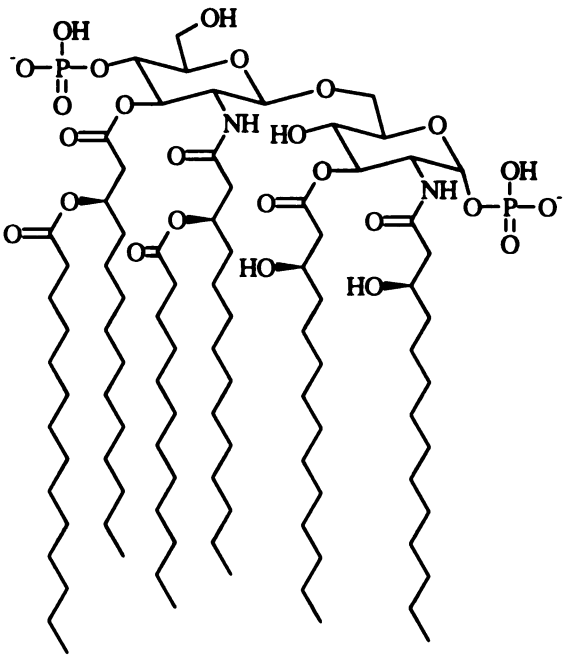

Figure 1.9: Structure of *E. coli* lipid A.

The lipid A moiety of LPS anchors it to the outer membrane of Gram-negative bacteria where it helps to form a barrier to environmental stress (77). It is the physical properties of the LPS aggregate, especially in the lipid A region, which provides it with this ability. Labischinski et al. (78) demonstrated that lipid A readily formed aggregates with a remarkable degree of order. They postulated that this aggregate order imparts the outer membrane with its ability to act as a permeability barrier. The ability to form highly ordered aggregates is a result of the three-dimensional conformation of lipid A and its ability to cross-link adjacent phosphates. Wang and Hollingsworth (79) have shown that lipid A adopts a compact triangular wedge shape in which the acyl chains are rigid and tightly packed together. This shape can readily pack into hexagonal arrays and explains the crystal structures observed (80, 81). Additional molecular order is provided from the

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cross-linking of adjacent phosphates by divalent cations. Coughlin et al. (75) found that this results in a dramatic increase in the rigidity of the lipid A headgroups.

Mechanisms of Lipopolysaccharide Induced Biological Activities

The broad spectrum of biological activities produced by LPS (see Table 1) and its clinical significance has produced a tremendous amount of interest in determining its mechanism of action. Studies by Michaleck et al. (82) demonstrated that many of the responses to LPS are mediated by immune cells. They used a mouse strain that is unresponsive to LPS (C3H/HeJ) and showed that LPS sensitivity could be restored following strong irradiation by an adoptive transfer of bone marrow cells from LPS responsive mice (C3H/HeN). They also found that LPS responsive mice could be rendered LPS unresponsive by the adoptive transfer of C3H/HeJ bone marrow cells. These results implicated bone marrow derived cells, such as lymphocytes and macrophages, in mediating the biological affects induced by LPS.

It is now known that LPS affects (activates) many animal cells including platelets, endothelial cells, B-lymphocytes and macrophages. The strong response of macrophages to LPS has made it the center of much research and has implicated it as the most important cell in mediating the response to LPS. Macrophages produce several soluble mediators (cytokines) when stimulated by LPS. These include interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), interferon (IFN), colony stimulating factor (CSF), prostaglandins and more (83). These mediators, in turn, modulate the response of other immune cells in a complex manner. The immune response is believed to be responsible for the physiological effects of LPS such as fever, hypotension, shock and death. This is supported by studies that demonstrated that the soluble mediators released by

macrophages are capable of eliciting biological effects that are associated with LPS (85-88).

Determining the biologically active moiety of LPS was of primary importance in understanding its mechanism. A breakthrough came in 1967 when Kasai and Nowotny discovered a *Salmonella minnesota* mutant that only synthesized a very short polysaccharide region (89). This mutant LPS still maintained all the biological activity of the wild type LPS. Thus, lipid A was suspected of being the active moiety. Imoto et al. (73) confirmed this when they synthesized lipid A and found that it contained nearly equivalent biological activity as LPS. A study by Haeffner-Cavaillon et al. (90) has modified this last assertion by demonstrating that the induction of IL-1 secretion was more effective with the 3-deoxy-D-*manno*-2-octulosonic acid (KDO) residues on the lipid A. While the proximal sugar residues are able to modulate its activity, the lipid A moiety is generally recognized to contain most of the biological activities of LPS.

With the discovery of lipid A as the biologically active moiety of LPS came a tremendous amount of interest in unraveling its structure-function relationship. This topic has been the focus of many studies that utilized natural and synthetic lipid A, lipid A analogues and partial structures. Results from these studies have been summarized in several excellent reviews (91-94). Among all the structures tested, it is the lipid A structure found in *E. coli* (Figure 1.9) that has optimal biological activity. Both the headgroup and fatty acid structures are critical components of this activity. Removal of either one of the phosphate groups results in a dramatic loss of activity. Loss of both phosphates renders lipid A inactive. The biological activities of lipid A are also dependent on the fatty acid structures. Addition or loss of one fatty acid, a change in the type of fatty acids, or the rearrangement of their positions results in a decrease in activity.

The sensitivity of the fatty acid structures is, perhaps, most apparent with the lipid A from *Rhodobacter sphaeroides*. This lipid A has the same headgroup structure as found in *E. coli* and differs only in its fatty acid region (Figure 1.10) (95). However, this lipid A is completely devoid of biological activity (95-99). A complete analysis of all the structure-function studies led Reitschel et al. (94) to conclude that the headgroup structures, especially the phosphates, are essential for binding of lipid A to immune cells, while the fatty acid region is responsible for the activation of the cells. Furthermore, they conclude that "the binding of lipid A is a necessary but not sufficient event" for the activation of immune cells, thereby implicating the hydrophobic interactions of the acyl chains as a critical component in eliciting the signal which results in cellular activation.

The search for the molecular mechanism of lipid A-mediated activation of immune cells has led to the proposal of specific membrane associated protein receptors (for reviews, see ref. 100-102). This was fueled by the finding that *Rhodobacter sphaeroides* lipid A is able to block the biological activities of enteric lipid A or LPS, suggesting that it competes for a specific binding site (97-99). Lei and Morrison (103, 104) detected an 80 kDa membrane-associated protein on B-lymphocytes, T-lymphocytes and macrophages by a photoactivatable LPS derivative. Hampton et al. (105) found that several proteins from a macrophage-like cell line bound to lipid A, of which a 31 kDa and a 95 kDa protein were predominant. Another report utilizing a macrophage-like cell line identified a 55 kDa and a 65 kDa protein that bound to lipid A (106). Kirkland et al. (107) found that 18 kDa, 25 kDa, and 28 kDa proteins from a B-lymphocyte cell line bound to lipid A. A report by Wright and Jong (108) implicated CD11/CD18 in the binding of LPS to macrophages. Finally, the LPS binding protein (LBP)/CD14 complex was shown to bind to LPS (109).

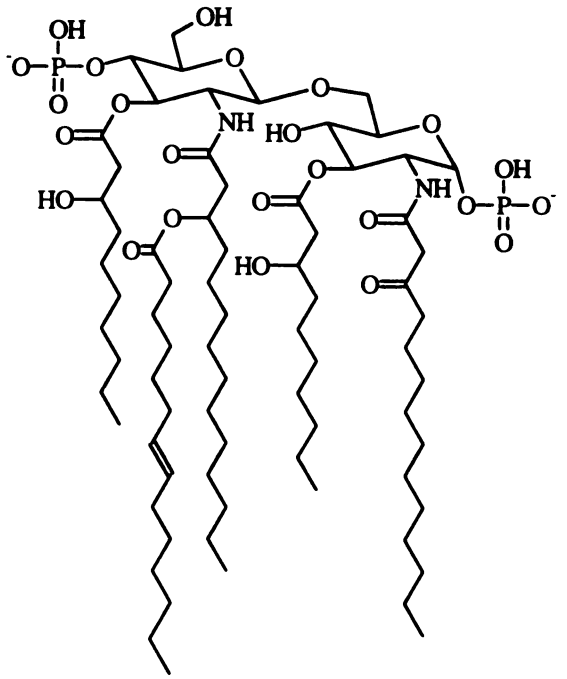

Figure 1.10: Lipid A structure from *Rhodobacter sphaeroides*.

To date, the best characterized of the putative LPS receptors is the LBP/CD14 complex. Studies suggest a sequence of events that lead to cellular activation by LBP/CD14 (see ref. 110). First, LBP, a soluble serum protein, binds to LPS. The LBP-LPS complex then is recognized by CD14 and the LPS is transferred to it. Finally, the LPS-CD14 complex elicits a signal transduction cascade. However, CD14 is not a transmembrane protein and is only loosely associated with the membrane via a phospholipid anchor (111). Therefore, another membrane protein, which interacts with LPS or LPS-CD14, is thought to be involved. A recent report suggests that this may be an 80 kDa protein (112). It is noteworthy, however, that LPS is capable of activating macrophages in the absence of LBP or CD14 (109). This is supported by the finding that

94% of CD14 could be removed from macrophages by phorbol esters without blocking their ability to respond to LPS (113). Another report showed that monocytes from CD14-deficient patients were able to produce IL-1 in response to LPS (114). Therefore, the role of CD14 in mediating an immune response to LPS is still undetermined. In fact, of all of the putative LPS receptors identified, none of them have been definitively shown to be responsible for the activation of immune cells.

While the role of LPS receptors remains unclear, it is clear that the lipid A region of LPS interacts non-specifically with membranes. Because lipid A is an amphiphilic molecule like any other lipid, it is obvious that it will be able to interact with and insert into membranes. There have been numerous reports illustrating the non-specific hydrophobic interaction of LPS with phospholipid, erythrocyte, platelet, lymphocyte, and macrophage membranes (see ref. 115). A few of these reports provide more direct support for the non-specific interaction of LPS with membranes. Schuster et al. (116) showed that LPS perturbed cholesterol-lecithin bilayer membranes (black lipid membranes). They introduced LPS into the aqueous phase next to the membrane and observed a rapid change in the membrane electrical resistance and stability. Their results suggest that the lipid A region of LPS inserted into the bilayer and caused a dramatic change in its integrity. Experiments by Jackson et al. (117) support the role of lipid A in binding to membranes. They attached spin labels to the carbohydrate groups of LPS and monitored its interaction with macrophages by electron spin resonance. The rate of motion of the labeled carbohydrates was not affected by the binding of LPS to macrophage cells, thus showing that the polysaccharide region of LPS is not involved in the binding to the macrophage membrane. Furthermore, this implicates lipid A as the region that is responsible for the binding. Experiments by Carr and Morrison (118, 119)

used polymyxin B (a small polycationic peptide) to analyze the binding of LPS to erythrocyte membranes. They demonstrated that the interaction of LPS with erythrocyte membranes was time and temperature dependent and occurred in two discernable steps. The first step is the ionic interaction of LPS with the membrane and can be prevented by the preincubation with polymyxin B. The second step is temperature dependent and was proposed by the authors to be due to the insertion of lipid A into the membrane. Jacobs and co-workers obtained nearly identical results when they studied the interaction of LPS with lymphocyte membranes (120). They also proposed that this interaction occurred in two steps with the first step being ionic in nature and the second being the irreversible insertion of lipid A into the membrane.

In 1987, Jacobs and Price (121) proposed a model to explain the molecular mechanism of the interaction of LPS with immune cells which leads to its activation (Figure 1.11). This model was formulated from an analysis of data from their lab as well as published data. This 'two-step' model is similar to that discussed above (118-120) and attempts to bring together the conflicting data on the role of LPS receptors and non-specific membrane interactions. The first step is the electrostatic interaction of lipid A headgroup and/or LPS polysaccharide region with the surface of the membrane. This is supported by the structure-function studies discussed above which concluded that the lipid A headgroup is involved in the binding to immune cells. The second step is the rearrangement of the LPS so that the lipid A is inserted (fused) into the membrane bilayer. This involves non-specific interactions of lipid A with the membrane components. This step is temperature dependent due to the change in the physical properties of the membrane and the LPS aggregates with temperature, thus affecting the

Figure 1.11: A schematic diagram of the two-step model proposed by Jacobs and Price (121). The first step is the electrostatic interaction of LPS with the cellular membrane. The second step is the insertion (fusion) of the lipid A region of LPS into the membrane. The insertion of LPS into the membrane is proposed to be the critical event leading to the activation of the immune cell. The LPS aggregate is (shown as a monolayer) likely to be a bilayer of LPS or LPS, lipids, and proteins depending on the source and purity of the LPS used.

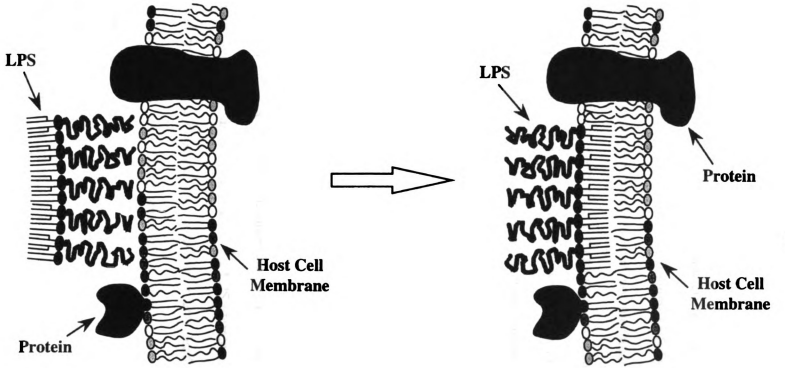
LPS
Host Cell
Membrane
Protein
LPS
Protein
Host Cell
Membrane
Step 1: Electrostatic Interaction
Step 2: Insertion

Figure 1.11

ability of lipid A to fuse with it. The authors propose that there may be proteins (or other molecules) which facilitate the binding and insertion of LPS into membranes. These molecules would have the properties characteristic of lipid A/LPS receptors. Furthermore, they propose that the activation of immune cells by LPS is a consequence of the insertion of lipid A into the membrane and not the binding to the surface of the membrane. The authors suggest that a membrane perturbation resulting from the insertion of lipid A may be the molecular signal which leads to cellular activation (122). This notion is supported by the structure-function studies (discussed above) that concluded that the acyl chain region of lipid A is responsible for the activation of immune cells. To this end, Jacobs and co-workers have demonstrated that the insertion of lipid A results in a decrease in the 'fluidity' of membranes (122-124).

Conclusion

To date, the two-step model proposed by Jacobs and Price (121) is the only molecular model for the activation of immune cells by LPS. Its strength lies in its ability to explain the seemingly disconnected data in the LPS literature. This model sparks new questions in trying to understand the molecular events leading to the activation of immune cells by LPS, and, more importantly, provides a framework to interpret experimental results.

In this dissertation, I explore the molecular events of LPS-cell interactions, specifically at the membrane level. The second chapter analyzes this interaction by utilizing the unusual LPS structures from *Rhizobium*. *Rhizobium* are soil bacteria that are not animal pathogens. However, they synthesize unusual LPS/lipid A structures and are useful in dissecting the LPS-host interactions. In the third chapter, I focus on the effects

that LPS/lipid A has on membranes. Since membrane properties are a very important factor in the function of membranes and cells, membrane perturbations elicited by LPS may be the critical molecular signal leading towards the activation of immune cells. I test this hypothesis and further our understandings of the non-specific LPS-membrane interactions. In the fourth chapter, I explore the role of membrane perturbations in mediating the biological effects of LPS. This is accomplished through the use of synthetic lipid 'probes'. Finally, the results obtained in this dissertation and their significance are summarized in Chapter 5.

The

Chapter 2

The Relationship Between Structure and Biological Activities of Lipopolysaccharides: Lessons from Rhizobial LPS

Introduction

Lipopolysaccharides (LPS) elicit a wide spectrum of biological responses in animals (see Table 1.1). It is now well established that most of the biological activities associated with LPS are due to the lipid A moiety. Many of these activities are the result of the interaction of lipid A with immune cells such as macrophages and B-lymphocytes. This interaction results in the alteration of the physiological state of the immune cell (usually to a more active state) and induces, for instance, the secretion of tumor necrosis factor-α (TNF-α) from macrophages and the increased cell division in B-lymphocytes. However, the molecular mechanism of the lipid A-host cell (or LPS-host cell) interaction and the physical basis for the mechanism of the modulation of immune cell activity is poorly understood.

Several researchers have proposed that the electrostatic interaction of lipid A with the cell membrane is the first step in the association of lipid A with immune cells (119-121). This interaction is thought to be mediated by the headgroup of lipid A, especially the phosphate groups (94). It is not clear whether the electrostatic interaction between lipid A and the immune cell is mediated by non-specific membrane interactions or is mediated by a stereo-specific lipid A receptor. Many investigators have proposed the existence of a receptor on the cell surface (see Chapter 1). However, no signal transduction events resulting from these interactions have been uncovered. Therefore, the role of a lipid A-specific receptor in the activation of immune cells remains to be determined.

The physicochemical properties of lipid A have been implicated as important determinants of biological activity. Using defined salt forms of LPS, Galanos and co-

workers (125, 126) demonstrated that its biological activity is very dependent on its salt form. Changes in the salt form of LPS are known to change its physical properties as well as its supramolecular (aggregate) size and structure (76, 125-128). Brandenburg and co-workers (7, 129) used Fourier transform infrared spectroscopy and X-ray diffraction to analyze the supramolecular structure of LPS. They found a strong correlation between the supramolecular conformation of LPS and its biological activity. These investigations have led to the proposal of an 'endotoxic conformation' as a determinant of its biological activity. The requirement of a particular supramolecular structure of LPS for the maintenance of biological activity is appealing since it explains how subtle changes in the lipid A headgroup or acyl chain structure, which might alter the supramolecular structure, can alter biological activity. It also opens up the possibility that other unusual lipid A structures can be biologically active provided that they have the proper physicochemical properties.

Of course, knowledge of the structures of LPS required for biological activity is a prerequisite to obtaining a detailed molecular understanding of the LPS-host cell interaction and the subsequent events that lead to cellular activation. Many studies have addressed this issue (see ref. 91-94). More recently, some very unusual lipid A structures have been identified from species of *Rhizobium*. Rhizobial LPS have been found to be distinct from enteric LPS in that they have a low phosphorous content (130) and contain an unusual trans-membrane fatty acid, 27-hydroxyoctacosanoic acid (131). The structure of *R. trifolii* lipid A was recently solved (Figure 2.1) (132). Its headgroup is comprised of a galacturonic acid 1(α)-6 linked to glucosamine. It does not contain phosphates. A lactic acid is glycosidically linked to the glucosamine. It also contains a total of five fatty

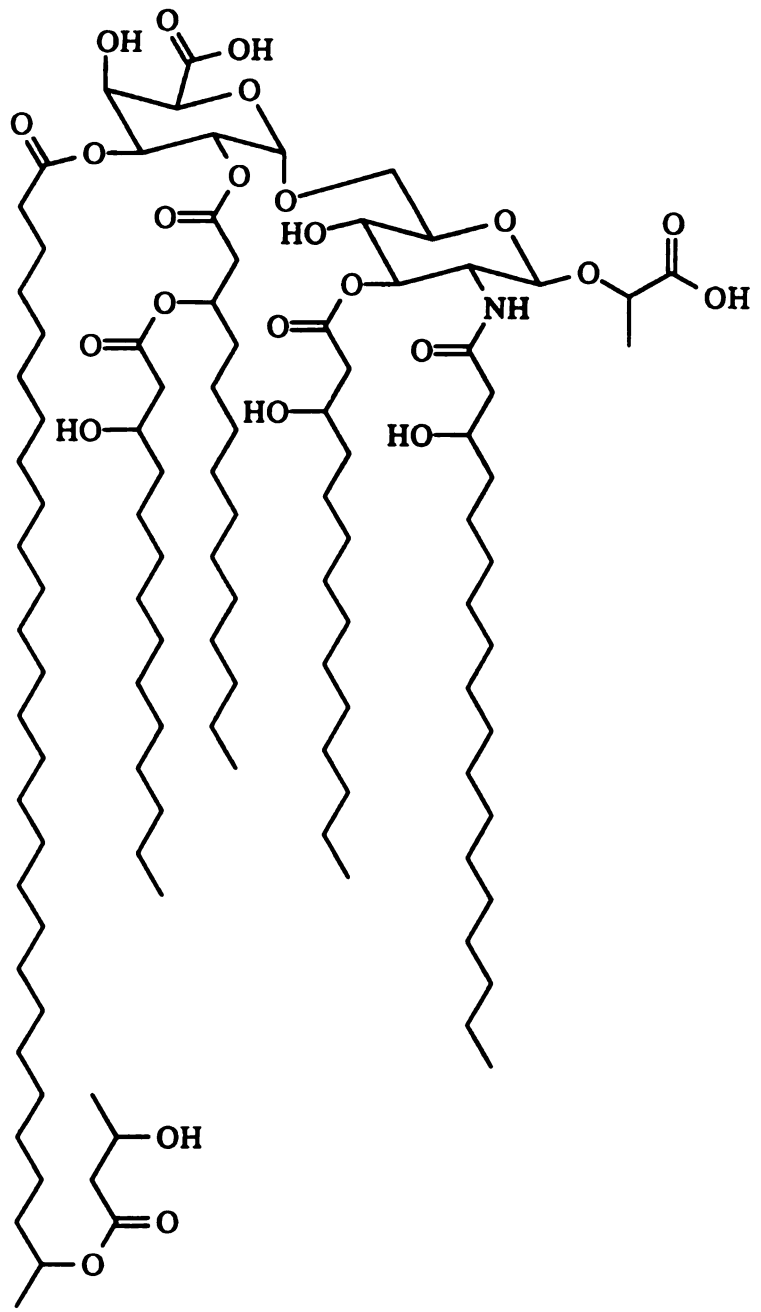

Figure 2.1: Structure of *R. trifolii* lipid A. From reference 132.

acids, four of which are attached directly to the headgroup carbohydrates at the 2, 3, 2', and 3' positions, including the 27-hydroxyoctulosonic acid (27-OH 28:0). Interestingly, this LPS still has some biological activity despite its unusual structure (133). One report also found that the lipid A from *R. meliloti* has an unusual structure although it retains the same headgroup as found in enteric bacteria (134). These authors report that it only contains four fatty acids including a 27-OH 28:0. This LPS has been shown to be about as biologically active as *E. coli* LPS (135). However, as will be discussed later in this chapter, studies in our laboratory on LPS on a different strain of *R. meliloti* do not agree with these reports.

The unusual lipid A structures found in species of *Rhizobium* provide an opportunity to analyze the LPS-cell interaction event and gain an insight into the molecular mechanisms by which LPS activates immune cells. In this chapter we describe the structural analysis of *R. meliloti* 2011 LPS and its biological activities. These results are compared to *R. trifolii* LPS and *Salmonella typhimurium* LPS in an effort to understand the structural features of lipid A that permit biological activity. In turn, these results allow us to make important conclusions about the molecular mechanisms of the LPS-cell interactions and the nature of the events that lead to cellular activation.

Materials and Methods

LPS Isolation and Purification. Lipopolysaccharide from *Salmonella typhimurium* (Sigma) was purified by gel filtration chromatography on a Sepharose 4B column using 0.05M ammonium formate, pH 5.5, as the buffer. The first peak eluting off the column was positive for 3-deoxy-D-*manno*-octulosonic acid (136) and was pooled,

dialyzed against distilled H_2O and lyophilized. *Rhizobium meliloti* 2011 was grown in modified Bergensen's medium (pH 6.9) and harvested in late exponential phase. Cells were harvested by centrifugation at 8,000Xg for 15 min. The cell pellet was vigorously stirred for 12 hrs with a water:chloroform:methanol:butanol (4:2:1:1) mixture at room temperature. After centrifugation, the aqueous phase was removed and extracted sequentially with chloroform and butanol. The aqueous phase (containing LPS) was treated with a solution of DNase I, RNase A, and $MgCl_2$ to remove contaminating nucleic acids. *R. meliloti* 2011 LPS was further purified by gel filtration chromatography on a Sepharose 4B column as described above. Column fractions were monitored for total carbohydrate content by the phenol-sulfuric acid assay (137), 3-deoxy-D-*manno*-2-octulosonic acid (KDO) by the periodate/thiobarbituric acid assay (136), uronic acids by the *m*-phenylphenol assay (138) and phosphorus by the method of Lowry et al. (139). The LPS was located in the carbohydrate peak that contained large amounts of KDO and no phosphate or uronic acids. LPS was dialyzed against distilled water, concentrated by rotary evaporation, and lyophilized. Cells used for ^{35}S labeling studies were grown under the same conditions with ^{35}S-sulfate supplemented in the media. Column fractions obtained from the extract of cells grown in ^{35}S enriched media were further assayed by scintillation counting. *Rhizobium trifolii* ANU843 was grown in modified Bergensen's medium (pH 6.9) and harvested at late exponential phase as described above. *R. trifolii* ANU843 LPS was extracted by aqueous phenol as previously described (140). The resulting LPS solution was dialyzed extensively against distilled H_2O, treated with DNase I and RNase A, and purified by gel filtration chromatography as described above. Galbraith Laboratories, Knoxville, TN, performed elemental analysis of LPS.

Lipid A Isolation. *R. meliloti* 2011 LPS was hydrolyzed with 5 ml of 1% acetic acid at 100°C for 2 hrs, cooled to room temperature, and partitioned between water and chloroform. The organic layer, which contained the lipid A, was dried under nitrogen.

Structural Analysis of Lipid A. *R. meliloti* 2011 lipid A was hydrolyzed with 1 ml of 5% HCl in methanol for 30 hrs at 70°C. The reaction mixture was cooled to room temperature and dried under a stream of nitrogen. The product was partitioned between water and chloroform, and the organic phase containing fatty acid methyl esters was concentrated to dryness under nitrogen. The fatty acid methyl esters were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). GC was performed on a Hewlett Packard 5890 instrument equipped with a capillary DB1 column (J & W Scientific, Folsom, CA) using a temperature program of 150°C to 300°C at 3°C/min, hold at 300°C for 20 min. GC/MS was performed on a Jeol JMS-AX505H spectrometer in the electron impact (EI) mode at 70 eV. The methyl glycosides in the aqueous phase were purified by loading the reaction mixture on a C18 cartridge (Sep-Pak, Waters Associates, Inc., Milford, MA) and eluting with 4:1 water:methanol. The purified methyl glycosides were peracetylated by a 1:1 mixture of pyridine:acetic anhydride (0.2 ml) for 12 hrs at room temperature. After peracetylation, the reaction mixture was acidified by the addition of 1 drop of 3M HCl, and partitioned between 0.5M NaCl and chloroform. The peracetylated methyl glycosides were recovered in the organic layer and concentrated under nitrogen. The sample was subjected to GC (capillary DB225 column, from 180°C to 230°C at 2°C/min, hold at 230°C for 80 min) and GC/MS analysis as described above.

Spectroscopy. Nuclear magnetic resonance (NMR) spectroscopy of *R. meliloti* lipid A was performed using the Varian VXR-500S (500 MHz) spectrometer in $CDCl_3$ with a small amount of CD_3OD added. Spectra were recorded at room temperature and were referenced to the chloroform solvent signal at 7.24 ppm. ^{31}P-NMR spectra were obtained on a Varian VXR-300 spectrometer.

USP Pyrogen Assay. The ability of *S. typhimurium*, *R. meliloti* 2011, and *R. trifolii* ANU843 lipopolysaccharides to induce fever in rabbits was determined according to the standard USP pyrogen protocol at the Biological Test Center, Irvine, CA. *S. typhimurium* LPS was administered at 0.05 μg/kg body weight. *R. meliloti* and *R. trifolii* LPS was administered individually at 0.05 μg/kg and 1.0 μg/kg. Body temperature was measured before and at 1, 2, and 3 hrs after injection and the maximum rise in temperature was used. If one or more rabbits exhibited a temperature rise of 0.6°C or greater or if the total temperature rise of the three rabbits exceeded 1.4°C, the test was considered to be positive.

USP Systemic Toxicity. The toxicity of *S. typhimurium*, *R. meliloti*, and *R. trifolii* LPS was determined in 5 mice. Tests were performed at the Biological Test Center, Irvine, CA. LPS was administered intraperitoneally at 500 or 1000 μg/animal. These animals were observed at 0, 0.25, 4, 24, 48, and 72 hrs. Weights of the mice were determined immediately before and after each trial. The sample is toxic if one of the following occurred: two or more mice died, abnormal behavior in two or more mice, or weight loss of more than 2 g in three or more mice.

Cell Lines. RAW264.7, a murine Abelson leukemia virus-transformed lymphocyte with macrophage properties (141), was obtained from American Type

Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100mg/L streptomycin sulfate (culture medium). Subcultures were prepared by scraping and resuspending in fresh culture medium. Cells were grown at 37°C in a humidified, 5% CO_2 atmosphere.

Tumor Necrosis Factor-α Assay. LPS samples were lyophilized, rehydrated in Dulbecco's PBS, sonicated, and vortexed thoroughly. RAW264.7 cells (2×10^5) were placed in 1ml of culture medium in 24 well culture plates and allowed to grow for 15 hrs. Samples (50 µl) were added to the cell culture wells and allowed to incubate with the cells at 37°C, 5% CO_2. Cells were allowed to incubate with the samples for 8 hrs since a time course experiment determined that the maximal TNF response occurred at this time. After incubation, cell culture supernatants were removed, centrifuged to remove cell debris, and placed on ice. The TNF-α concentration in the culture supernatant was determined using a mouse TNF-α ELISA kit (Genzyme Corp., Cambridge, MA). TNF-α levels were quantitated with recombinant mouse TNF-α according to the test kit instructions. The results shown are the average of duplicate determinations and the error bars show the range of the results.

Limulus amebocyte lysate (LAL) assay. The LAL assay was performed with the Whittaker Bioproducts QCL-1000 Quantitative Chromogenic LAL, Whittaker Bioproducts, Inc. (Walkersville, MD). All assays were standardized to Endotoxin Units (EU) using *E. coli* 0111:B4 LPS according to the assay instructions. Samples were prepared in distilled water and then sonicated and vortexed thoroughly immediately prior to being assayed. The concentration of LPS tested was 0.1 ng/ml. The assay was

performed following the test kit instructions. Results shown are the mean of at least three determinations.

Results

Rhizobium meliloti 2011 Lipopolysaccharide isolation and characterization. LPS from *R. meliloti* was extracted and purified by gel filtration chromatography (Figure 2.2). LPS was contained in the first carbohydrate peak that eluted off the column. KDO, a common LPS sugar, was detected only in this peak. This peak contained only trace levels of uronic acids or phosphate, indicating that these are not major components of the LPS. Also, no signal was detected by ^{31}P-NMR spectroscopy.

Interestingly, *R. meliloti* cells grown in ^{35}S-sulfate enriched medium were found to incorporate ^{35}S into their LPS (Figure 2.3). The ^{35}S is thought to be incorporated as sulfate groups since it is readily removed by mild acid hydrolysis and the liberated sulfate can be precipitated by Barium (Hollingsworth, unpublished results). Sulfate is a rare component in LPS and has previously only been reported for *Pseudomonas aeruginosa* LPS (142). A sulfur content of 0.5 % (w/w) for *R. meliloti* 2011 LPS was demonstrated by elemental analysis. Given an approximate LPS molecular weight of 10000 g/mole, this would be the equivalent of an average of 1.5 sulfate groups per LPS molecule. The locations of the sulfate groups are difficult to determine because they are so labile. However, chloroform extraction of bacterial cultures yielded free lipid A (without acid cleavage) which was found to contain ^{35}S, thus demonstrating that at least some of the sulfate groups are associated with the lipid A moiety (Hollingsworth, unpublished results).

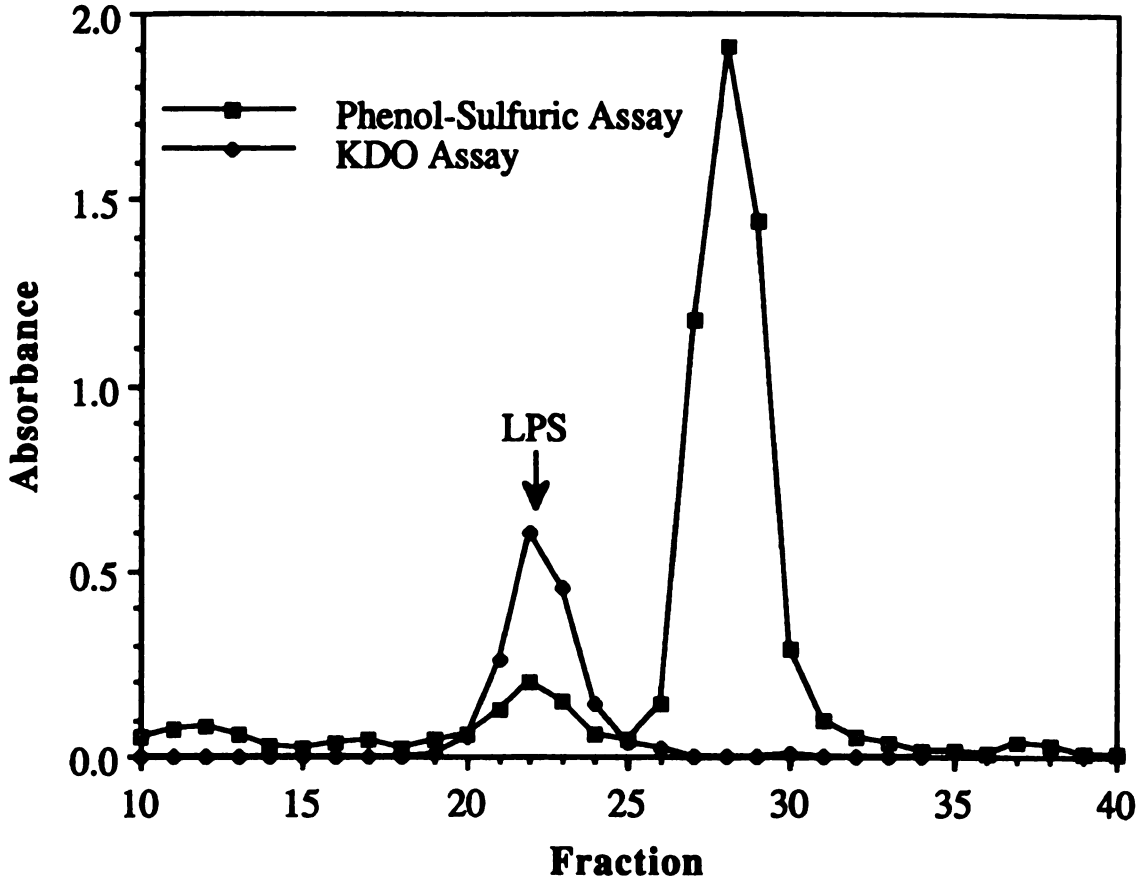

Figure 2.2: Purification of *R. meliloti* 2011 LPS by Sepharose 4B gel filtration chromatography.

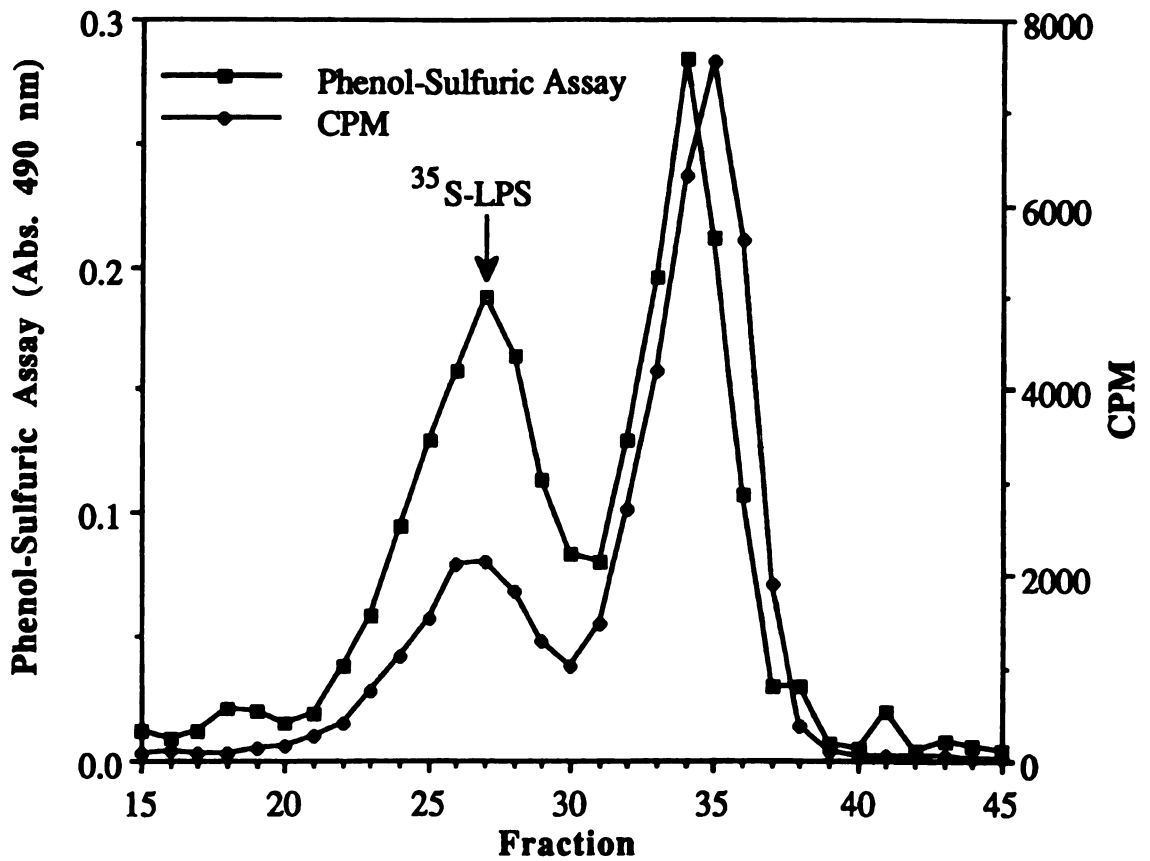

Figure 2.3: Purification of ^{35}S-labelled *R. meliloti* 2011 LPS by gel filtration chromatography.

R. meliloti 2011 lipid A was obtained by the mild acid hydrolysis of the LPS. Analysis of the carbohydrates contained in the lipid A was accomplished by gas chromatography (GC) and GC-mass spectrometry (GC-MS) (Figure 2.4). The results indicate that glucosamine is the major component. Some glucose and heptose were also identified. Although the possibility of glucose and heptose being an integral part of the lipid A structure can not be ruled out, they are common core and O-antigen oligosaccharide components and are, therefore, believed to be contaminants remaining from the acid hydrolysis of LPS. The fatty acids of *R. meliloti* lipid A were also analyzed by GC and GC-MS (Figure 2.5). Three fatty acids were predominant: 3-hydroxytetradecanoic acid (3-OH 14:0), 3-hydroxyoctadecanoic acid (3-OH 18:0), and 27-hydroxyoctulosonic acid (27-OH 28:0). They were present in an approximate molar ratio of 3:1:1, respectively. Hexadecanoic acid (16:0), 3-hydroxyhexadecanoic acid (3-OH 16:0) and octadecanoic acid (18:0) were present in approximate molar ratios of less than 0.4.

The ^{1}H-NMR spectrum of *R. meliloti* lipid A was acquired in a deuterated chloroform/methanol solvent. This yielded a high resolution spectrum (Figure 2.6). The very intense signal at 1.15 ppm and the triplet at 0.78 ppm are due to the methylene and terminal methyl protons of the acyl chains, respectively. The group of signals between 2.1 ppm and 2.6 ppm arise from protons on the acyl chain α to the carbonyl carbon. Some doublets of doublets (such as those at 2.29 ppm and 2.53 ppm) are resolved and are characteristic of the protons coupled to the methine proton at the 3 position of hydroxy fatty acids. The triplet at 2.19 ppm is most likely due to protons on the 2 position of 27-OH 28:0 since this is the primary fatty acid present without a substituent on the third

Figure 2.4: Gas chromatograph of the methylated and peracetylated carbohydrates from *R. meliloti* 2011 lipid A. The carbohydrates are A. two isomers of glucose, B. heptose, and C. glucosamine.

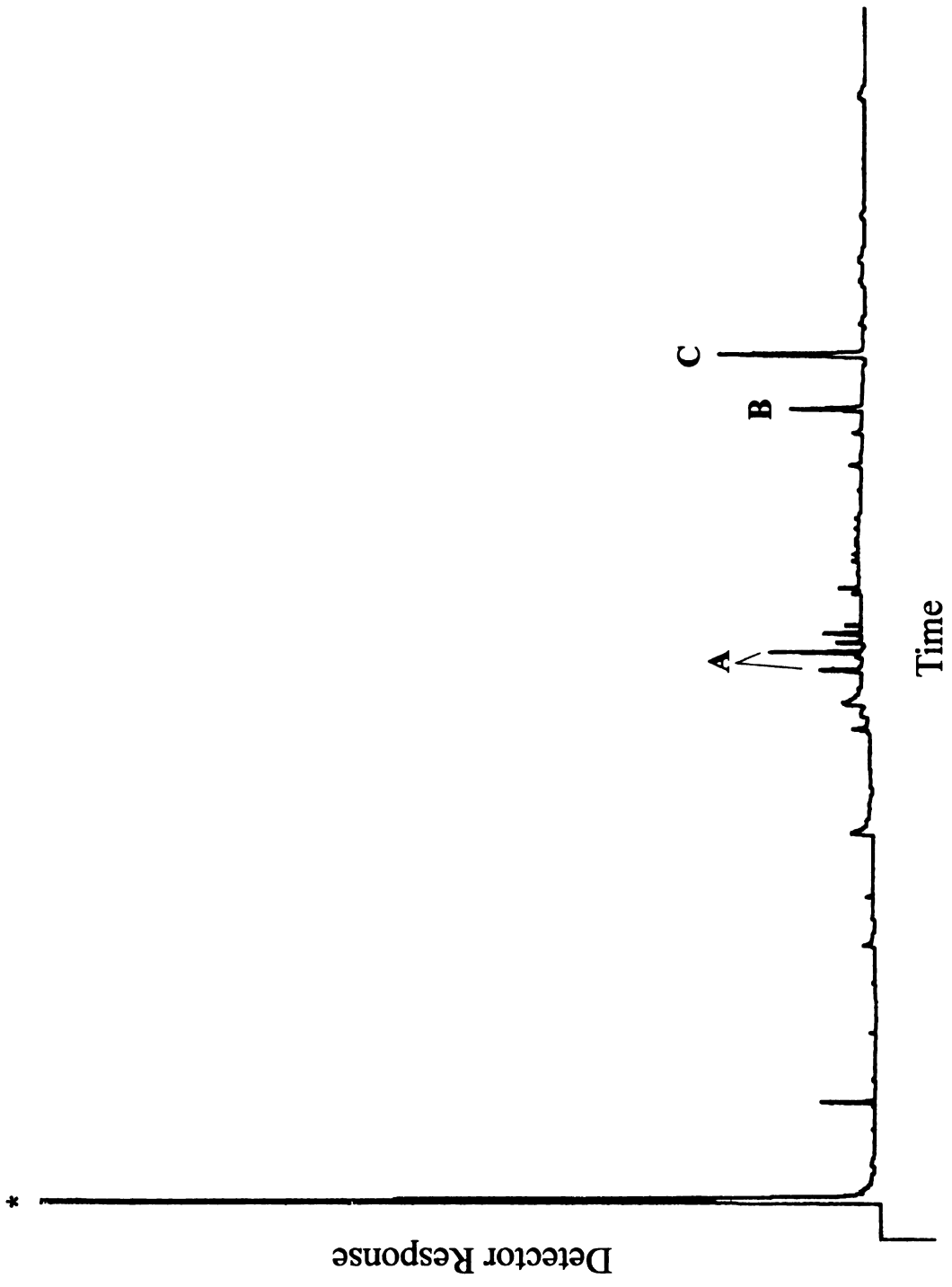

Figure 2.4

Figure 2.5: Gas chromatograph of the fatty acid methyl esters from *R. meliloti* 2011 lipid A. The peaks A-F correspond to 3-OH 14:0, 16:0, 3-OH 16:0, 18:0, 3-OH 18:0, and 27-OH 28:0, respectively.

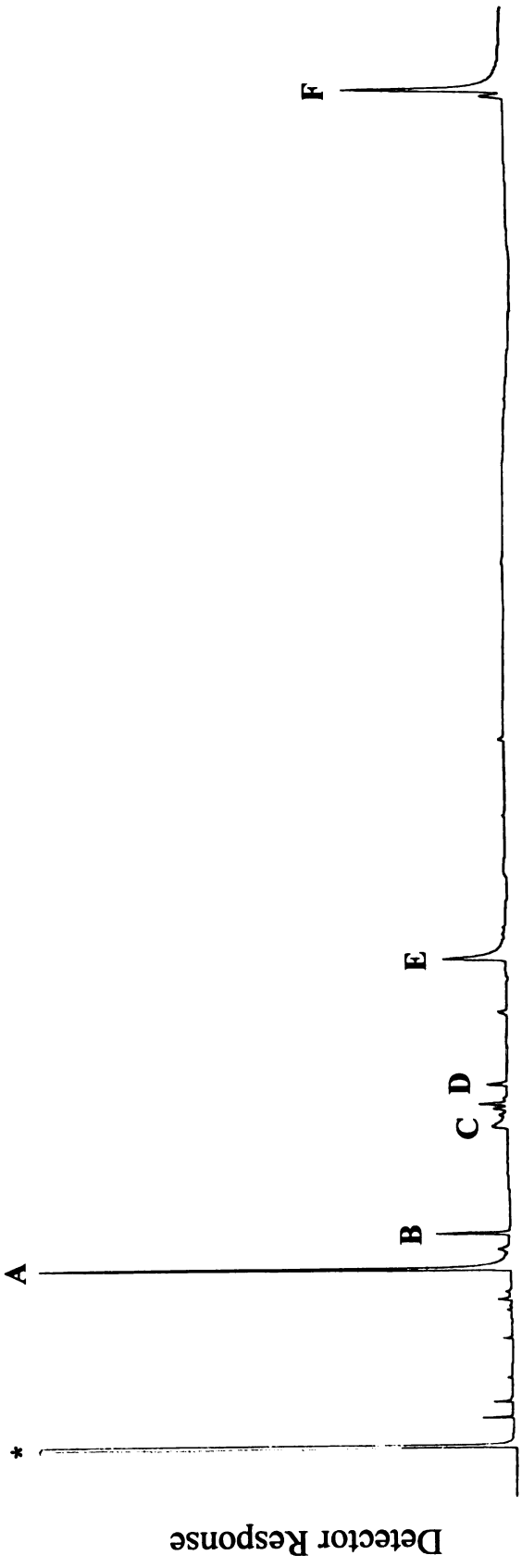

Figure 2.5

Figure 2.6: ^{1}H-NMR spectrum of *R. meliloti* 2011 lipid A. Spectral assignments are given in the text.

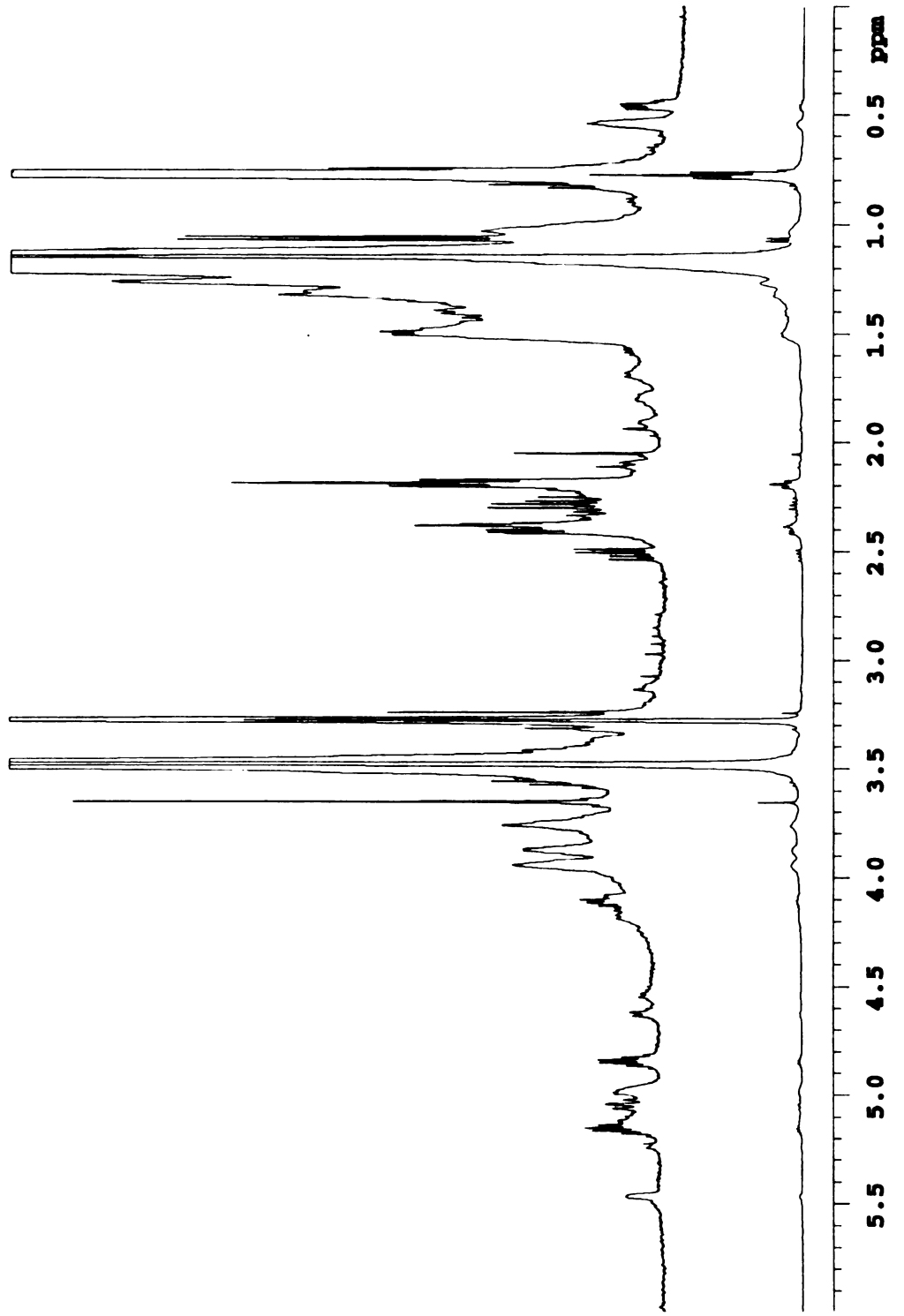

Figure 2.6

carbon. The complex multiplets between 1.2 ppm and 1.6 ppm are primarily due to methylene protons in the acyl chains that are α to the hydroxy-substituted carbons or are β to the carbonyl carbons. The intense signals at 3.28 ppm and 3.48 ppm are due to the deuterated methanol and residual water in the sample, respectively. The remaining signals between 3.0 ppm and 4.2 ppm are primarily from methine protons of the fatty acid carbons that bear an unsubstituted hydroxyl group and from protons on the carbohydrate headgroup. The group of signals between 4.5 ppm and 5.5 ppm are primarily from anomeric protons, protons located on the carbohydrate carbons that are acylated, and methine protons on the fatty acyl carbons that bear an acyloxyacyl substituent. Interestingly, the spectrum contains a quartet at 4.85 ppm and a doublet at 1.07 ppm. These signals are identical to the signals found in *R. trifolii* lipid A and assigned to a lactic acid group attached to the 1 position of the glucosamine residue (132). The presence of these signals in *R. meliloti* lipid A indicates that it also has this group.

Biological Activity of R. meliloti 2011 LPS. Four standard tests for biological activity were carried out on the LPS of *R. meliloti* 2011 and the results compared to *R. trifolii* LPS and *Salmonella typhimurium* LPS. The tests used to measure biological activity were (1) the USP pyrogen test in rabbits, (2) the systemic toxicity test in mice, (3) induction of tumor necrosis factor-α release from a macrophage cell line, and (4) the ability to activate the clotting enzyme cascade of the amebocyte lysate from *Limulus polyphemus* (LAL assay). With the exception of the tumor necrosis factor assay and LAL assay, the biological activities for *R. trifolii* LPS and *S. typhimurium* LPS were presented earlier (133) and are provided here for completeness.

The ability to induce the secretion of cytokines (immune mediators) from macrophages is a critical measure of the biological activity of LPS. Tumor necrosis factor-α (TNF-α) is one of the primary mediators of the biological effects of LPS and is the cytokine produced first by macrophages in response to LPS. In turn, TNF-α stimulates the secretion of other cytokines such as interleukin-1 (IL-1) (85). Together, TNF-α and IL-1 are thought to be the major mediators of the biological effects of LPS including the induction of fever and toxicity (85-88). We tested the ability of rhizobial and enteric LPS to induce the secretion of TNF-α from a macrophage-like cell line (RAW264.7) known to be activated by LPS (141). LPS from *R. trifolii* and *R. meliloti* were far less potent than that from *S. typhimurium* (Figure 2.7). At 1.0 ng/ml, *R. trifolii* LPS and *R. meliloti* LPS induced 6% and 0% of TNF-α compared to *S. typhimurium* LPS, respectively. Both of the rhizobial LPS were able to induce the production of TNF-α at higher concentrations.

Since it is generally thought that TNF-α is the primary mediator of pyrogenicity and toxicity induced by LPS, it would be predicted that rhizobial LPS would not be as effective as enteric LPS in these tests. This is indeed what was found. The toxicity of the LPS was tested in mice at 500 μg and 1000 μg LPS per animal (Table 2.1). The mice were monitored for signs of toxicity and their weights were recorded before and after the test as a measure of their health. The rhizobial LPS did show some signs of toxicity including lethargy and moderate weight loss. However, they were clearly far less toxic than *S. typhimurium* LPS. Interestingly, *R. meliloti* LPS appeared slightly more toxic than *R. trifolii* LPS. This is the opposite of what we would predict from the TNF induction assay. The ability of the LPS to induce fever in rabbits was tested (USP

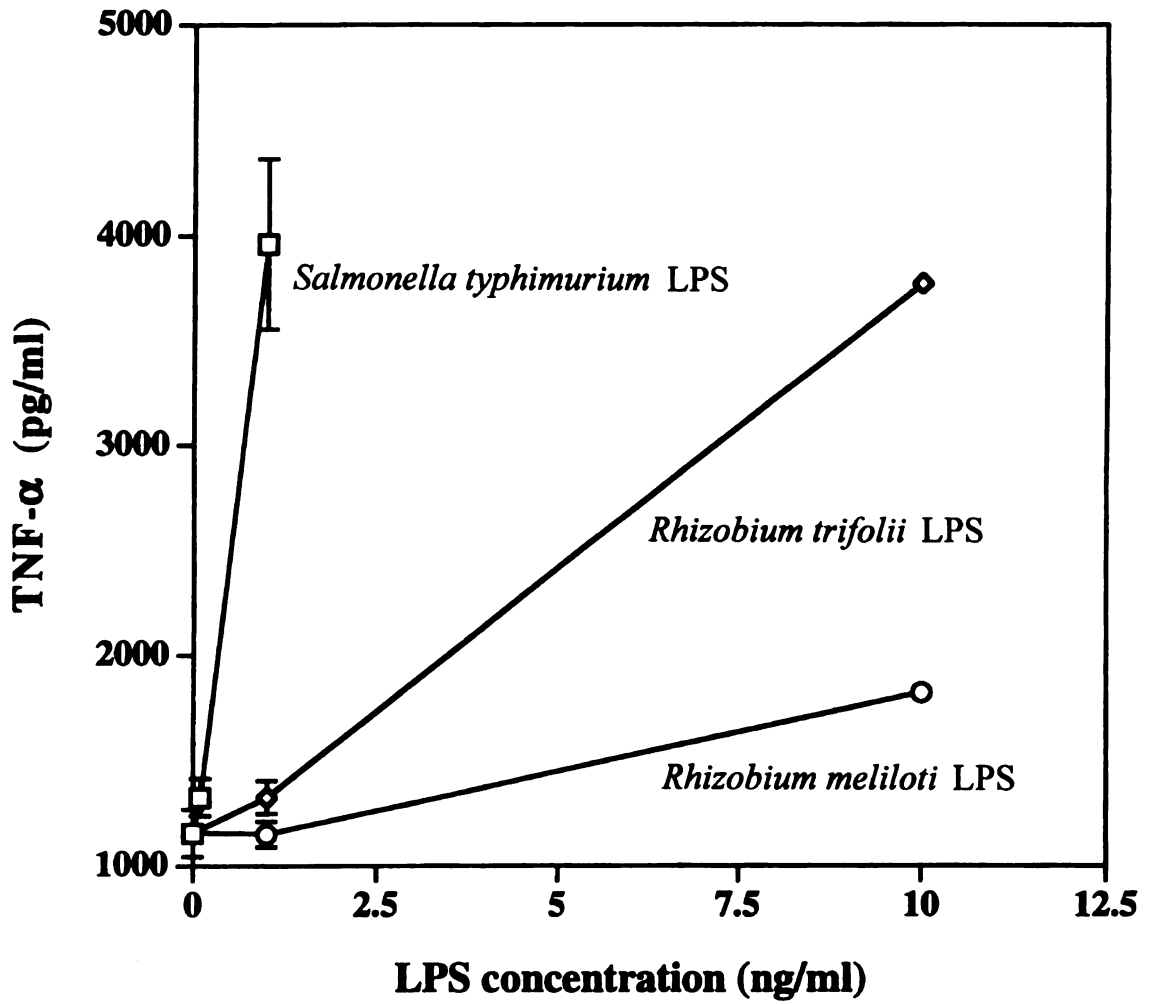

Figure 2.7: Tumor Necrosis Factor (TNF-α) assay of *S. typhimurium* LPS, *R. meliloti* 2011 LPS, and *R. trifolii* ANU843 LPS.

Table 2.1: Systemic toxicity of *S. typhimurium*, *R. meliloti* 2011, and *R. trifolii* ANU843 LPS.

Sample	Dose (µg)	Weight Before (g)	Observations[1] (hrs) 0	0.25	4	24	48	72	Weight After (g)
Salmonella typhimurium LPS	500	21	0	0	1	D	-	-	-
		20	0	0	1	D	-	-	-
		20	0	0	1	1	1	1	14
		19	0	0	1	1	1	1	15
		17	0	0	1	1	1	1	14
	1000	19	0	0	1	D	-	-	-
		21	0	0	1	D	-	-	-
		20	0	0	1	D	-	-	-
		22	0	0	1	D	-	-	-
		19	0	0	1	D	-	-	-
Rhizobium meliloti LPS	500	22	1	1	1	0	0	0	27
		21	1	1	1	0	0	0	22
		18	1	1	1	0	0	0	19
		20	1	1	1	0	0	0	20
		22	1	1	1	0	0	0	23
	1000	19	1	1	1	0	1	0	17
		23	1	1	1	0	1	0	22
		23	1	1	1	0	1	0	21
		22	1	1	1	0	1	0	18
		21	1	1	1	0	0	0	20
Rhizobium trifolii LPS	500	21	0	0	1	0	0	0	21
		21	0	0	1	0	0	0	23
		21	0	0	1	0	0	0	23
		22	0	0	1	0	0	0	24
		20	0	0	1	0	0	0	22
	1000	22	0	0	1	1	0	0	21
		20	0	0	1	1	0	0	20
		20	0	0	1	1	0	0	20
		18	0	0	1	1	0	0	19
		17	0	0	1	1	0	0	16

[1]Observations: 0 = no symptoms of toxicity, 1 = lethargy, and D = death

pyrogen assay). Again, the rhizobial LPS are less potent than *S. typhimurium* LPS in inducing fever (Table 2.2). *R. trifolii* LPS produced a significantly higher fever than *R. meliloti* LPS.

In 1964, Levin and Bang (143) reported that amebocytes obtained from the horseshoe crab (*Limulus polyphemus*) were extremely sensitive to LPS. LPS activates a clotting enzyme cascade causes the formation of gelatinous clot, or, if an artificial substrate is used, into the development of a color. This was developed into an *in vitro* assay to test the biological activity of LPS, called the *Limulus* amebocyte lysate (LAL) assay, and is widely accepted as a measure of its biological activity. The results for the LAL assay are shown in Figure 2.8. *S. typhimurium* LPS, *R. meliloti* LPS and *R. trifolii* LPS had an LAL activity of 7.8 EU/ng, 13.3 EU/ng and 13.9 EU/ng, respectively. Clearly, the rhizobial LPS are very active in this assay.

Discussion

The structural characterization of *Rhizobium meliloti* lipid A clearly demonstrates that it has an unusual structure compared to *E. coli* or *Salmonella* lipid A. Although we do not, at the present time, have its complete structure determined, we know that it is substantially different than enteric lipid A. The headgroup is composed primarily of glucosamine. This is consistent with a glucosamine disaccharide headgroup similar to that found previously for *R. meliloti* (134) and enteric lipid A. However, instead of phosphates, *R. meliloti* lipid A contains lactic acid and sulfate. The fatty acids present are also significantly different. *R. meliloti* lipid A contains fatty acids ranging from 14-28 carbons long, which is much longer than found in enteric lipid A. From the molar ratios

Table 2.2: USP Pyrogen assay of *S. typhimurium*, *R. meliloti* 2011, and *R. trifolii* ANU843 LPS.

Sample	**Dose** (μg/kg)	**Weight** (kg)	**Max. Temp. Rise** (°C)	**Total Temp. Rise** (°C)
Salmonella typhimurium	0.05	2.63	1.18	2.89
		3.26	1.13	
		2.74	0.58	
Rhizobium meliloti	0.05	1.88	0.00	0.06
		1.97	0.00	
		1.86	0.06	
	1.00	1.92	0.00	0.65
		1.69	0.28	
		1.77	0.37	
Rhizobium trifolii	0.05	1.91	0.30	1.56
		2.59	1.13	
		2.50	0.13	
	1.00	2.37	0.73	2.70
		2.15	1.19	
		2.24	0.78	

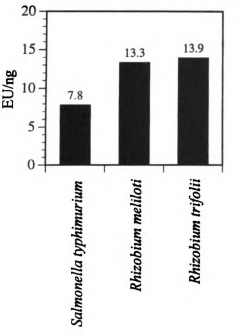

Figure 2.8: *Limulus* Amebocyte Lysate (LAL) assay of *S. typhimurium* LPS, *R. meliloti* 2011 LPS, and *R. trifolii* ANU843 LPS. Activity is given as Endotoxin Units (EU) per ng LPS.

of the fatty acids, *R. meliloti* lipid A appears to contain five fatty acids (three 3-OH 14:0, one 3-OH 18:0, and one 27-OH 28:0). Overall, the structure of *R. meliloti* lipid A and *R. trifolii* lipid A appear to be similar. They both contain a lactic acid, a disaccharide headgroup, and similar length and number of fatty acids. The major difference lies in the galacturonic acid and the lack of sulfate in the headgroup of *R. trifolii* lipid A.

The biological activities of the rhizobial LPS reflect these similarities. They both have similar levels of biological activities. Both are lower in toxicity than *S. typhimurium* LPS and are weaker in their ability to induce the production of TNF-α and induce fever. However, they still harbor these biological activities albeit at a lower level. Interestingly, both rhizobial LPS were very powerful stimulators of the LAL assay.

Furthermore, Lill-Elghanian (133) reported that *R. trifolii* LPS is a potent immune stimulant, being nearly as active as *Salmonella* LPS in stimulating the division of B-lymphocytes (B-cell mitogen assay).

Many earlier structure-function studies on lipid A have attempted to utilize its potentially beneficial biological activities (immune stimulation) while minimizing its toxic effects. Some success has been found with monophosphoryl lipid A (MPLA) (144). MPLA is very weakly toxic but is still active in the LAL assay and is a good B-lymphocyte mitogen (93). LPS from *R. meliloti* 2011 and *R. trifolii* ANU843 also appear to have these properties. They both have a low level of toxicity but are strong inducers of LAL activity. Therefore, they may also have some therapeutic value similar to that found for MPLA. Interestingly, Lill-Elghanian reported that, unlike MPLA, *R. trifolii* LPS is not able to induce an antibody response from B-lymphocytes (133). The unusual structures of Rhizobial LPS, along with their distinct biological activities, reaffirms the notion that the biological activities elicited by LPS are not triggered by a single mechanism. Furthermore, it questions the role of a universal LPS receptor (such as LBP/CD14) in triggering the LPS-response of many different cell types including macrophages and B-lymphocytes.

The unusual lipid A structures from *R. meliloti* and *R. trifolii* provides an insight into the LPS-cell interaction. As mentioned above, this interaction is mediated by electrostatic interactions. It has been postulated that the binding of lipid A to a stereospecific receptor on the membrane surface induces a signal transduction cascade that elicits a physiological response (see Chapter 1). However, *R. meliloti* and *R. trifolii* LPS are able to elicit a physiological response even though they have completely

different charge functionalities than enteric lipid A. This clearly indicates that the LPS-cell interaction is not very specific. The lack of specificity does not necessarily rule out the possible involvement of membrane proteins. Indeed, the most promising of the putative LPS receptors (CD14) has been found to be very non-specific (145, 146). CD14 readily binds to a variety of polyanionic substances and is, therefore, not a lipid A-'*specific*' receptor. We feel that this lack of specificity severely questions a direct role of lipid A receptors in the activation of immune cells. Perhaps, as suggested in the two-step model (121), these receptors simply facilitate the incorporation of lipid A into the host cell membrane. In fact, several recent reports have demonstrated that LBP and CD14 facilitate the incorporation of LPS into phospholipid bilayers (147-149).

As discussed in Chapter 1, the hydrophobic interactions of the acyl chains of lipid A with the host cells is a critical component of eliciting a physiological response (94). The biological activity of lipid A is extremely sensitive to its fatty acid structure. This is apparent with the lipid A from *Rhodobacter sphaeroides* (Figure 1.10) that contains five fatty acids but is completely devoid of biological activity (95-99). However, I demonstrate here that the lipid A from *Rhizobium* contains the same number of fatty acids but retains some biological activity. Obviously, the number of fatty acids present *per se* is not the factor determining biological activity. The fatty acids must be influencing some property of the lipid A that is able to modulate its activity. We can begin to understand this by analyzing the differences between the fatty acyl structures found in enteric, *R. sphaeroides*, and *Rhizobium* lipid A. It is known that enteric lipid A readily forms highly ordered aggregates (78, 79). *R. sphaeroides* lipid A has only five short fatty acids, one of which is unsaturated. This would be expected to significantly decrease the

order of the fatty acyl region of this lipid A as well as decrease its aggregate order. This has recently been demonstrated by Bérubé (150). The lipid A from *Rhizobium* also has only five fatty acids; however, they are all saturated fatty acids and are much longer than found in enteric lipid A. This would be expected to increase the order of the fatty acyl region. This is especially true for the trans-membrane 27-OH 28:0 fatty acid found in *Rhizobium*. It has been demonstrated that trans-membrane fatty acids synthesized by thermophilic bacteria help to stabilize their membrane (151). This same effect would be expected for the 27-OH 28:0 fatty acid found in rhizobial lipid A. We believe that this change in the physicochemical properties of lipid A (especially in the order of the fatty acyl region) is responsible for the modulation of its biological activity.

As discussed in the introduction, the structure of lipid A from a different strain of *R. meliloti* (10406) has been reported (134). Its structure contains a glucosamine disaccharide backbone bearing two phosphates and four fatty acids. The fatty acids are of the same length as the *R. meliloti* 2011 lipid A structure reported here, including a 27-OH 28:0 fatty acid. Interestingly, LPS from *R. meliloti* 10406 was found to be very toxic (135). The different biological activity elicited by this LPS, as well as the structural studies, clearly shows that *R. meliloti* 10406 and *R. meliloti* 2011 lipid A have different structures. This is surprising since the structure of lipid A is usually invariant among closely related bacteria. However, because *R. meliloti* 10406 lipid A is proposed to have only four fatty acids and is still toxic, the studies on it only serve to strengthen the arguments made above.

In this study, I demonstrated that lipid A from *Rhizobium* have very unusual structures but still retain biological activity. These results clearly demonstrate that the

LPS-host cell interaction is not highly specific. They also indicate that the physicochemical properties of lipid A, especially in the fatty acyl region, is responsible for the activation of immune cells. This is in agreement with the studies cited above that found a correlation between the physicochemical properties of LPS and its biological activity (7, 125-129). While it is difficult to determine the underlying physical mechanism of this correlation, the importance of the order of the fatty acyl region of lipid A suggests that it may involve a perturbation in the properties of the host cell membrane.

Chapter 3

Lipopolysaccharide Induced Changes in the Order and Dynamics of Membranes

Introduction

Lipopolysaccharides (LPS) are complex glycolipids located on the outer membrane of Gram-negative bacteria. LPS are capable of eliciting a broad spectrum of biological activities in animals including fever, hypotension, shock and death. These effects are primarily due to the severe immune response induced by LPS.

The first interaction of LPS with the host (immune) cell occurs at the membrane. Since LPS are amphiphilic molecules, it is understandable that insertion of its lipid anchor (lipid A) into the host cell membrane should take place. Several early investigations suggested this mode of interaction (152-156). Since then, many reports have definitively demonstrated that the lipid A region of LPS inserts into membranes (see Chapter 1 and ref. 116-120, 157). Moreover, it has been proposed that the activation of immune cells by LPS requires the insertion of lipid A into the host cell membrane (117, 121, 158).

Although LPS have been the focus of intensive research for more than 20 years, the signal that initiates an LPS-induced cellular response has remained elusive. This signal could arise from perturbations in the host cell membrane properties that occur when LPS inserts. This is reasonable since it is known that the physical properties of membranes are sensitive regulators of their functions and, in turn, modulate cellular functions (see Chapter 1). The ability of LPS to form highly ordered, negatively charged aggregates (78, 79) suggests that it would be capable of inducing substantial perturbations in the properties of membranes in which it was incorporated. The requirement of the insertion of LPS into the host cell membrane to elicit a cellular response is consistent with the involvement of such perturbations. Moreover, the

dependence of the activity of LPS on its structure and physicochemical properties (detailed in Chapter 2) would also support a role of membrane perturbations. The aggregate form of LPS and its degree of acyl chain order would be especially critical to its ability to alter membrane properties and would explain why its fatty acid composition is so critical to its ability to activate immune cells (94).

The above arguments indicate that membrane perturbations might be a critical component in the activation of immune cells by LPS. However, relatively little is known about how LPS causes such perturbations. The first indirect evidence that the incorporation of LPS into phospholipids results in a change in its physical properties was provided by Rothfield and Takeshita (159). They demonstrated that phospholipid-LPS mixtures protected a transferase against thermal inactivation whereas neither LPS nor the phospholipids were effective alone. Since then, several other studies have investigated the effects of LPS on the dynamics and order of membranes. Lui et al. (160) showed that LPS increased the transition temperature of phosphatidylethanolamine and phosphatidic acid. This indicated that LPS increased the molecular order of these lipids. Their observation that LPS decreased the width of the phase transition (thus increasing the cooperativity) supports this conclusion. Rottem (161) found that LPS reduces the mobility of the acyl chains in membranes by using spin-labeled phospholipids and electron spin resonance. A series of studies by Jacobs and co-workers (121, 123, 124) support this result. Using fluorescence depolarization of a lipid probe, they demonstrated that LPS decrease the 'fluidity' of phospholipid membranes. Interestingly, an NMR spectroscopy study showed that LPS increased the acyl chain order of phospholipids in the liquid crystalline phase but decreased the acyl chain order in the lamellar-gel phase

(162). Other investigators have measured the degree of acyl chain motion of the membranes of intact macrophages (163, 164) and hepatocytes (165). These studies have also noted a decrease in the acyl chain motion of the cellular membranes in response to LPS. However, these changes were measured several hours after LPS was added. Therefore, the possibility that the change in the physiological state of the cell were responsible for these changes, and not LPS directly, could not be excluded. All of these studies support the general conclusion that LPS is capable of reducing the acyl chain motion and increasing the degree of order of lipids.

Other studies have analyzed the lateral order of LPS in phospholipids in an attempt to understand the organization of the outer membrane of Gram-negative bacteria. Fried and Rothfield (166) studied the changes in the equilibrium surface pressure of phospholipid monolayers induced by LPS. Their results were consistent with a homogeneous lipid-LPS mixture. A similar conclusion was reached in a ^{2}H-NMR spectroscopy study on LPS-phospholipid systems (162). However, reports by Takeuchi and Nikaido (167, 168) contradict these findings. Using electron spin resonance and spin-labeled phospholipids, they found that LPS aggregated together in stable domains in the plane of the membrane. The conflicting results from these investigations leave the issue of the lateral organization of phospholipid-LPS systems in question.

While the previous investigations on the effects of LPS on membrane properties have led to the general conclusion that LPS decreases the 'fluidity' of membranes, they provide only little molecular details of these changes. Additionally, all but one of these studies (162) used lipid probes to investigate the membrane properties. These results are limited to the region immediately around the probe and may not be representative of the

entire membrane. Furthermore, the effect of LPS on the lateral order of membranes needs to be addressed. In this report, I investigate the effect that LPS has on phosphatidylcholine model membranes. I use a variety of biophysical techniques to provide detailed information about how LPS affects the order and dynamics of membranes. These include fluorescence depolarization, x-ray diffraction and ^{1}H-NMR spectroscopy. Emphasis is placed on ^{1}H-NMR spectroscopy since this technique can look at specific regions within the membrane and can be used to understand the molecular environment around nuclei. NMR spectroscopy measures all the lipids in the membrane and is not limited to the environment around a lipid probe. Furthermore, spin-lattice relaxation times (T1) of the nuclei are sensitive to their degree of motion and are an excellent way to characterize the dynamic state of membranes. Through these techniques, I demonstrate that LPS dramatically affects both the order and the dynamics of membranes.

Materials and Methods

LPS Isolation and Purification. Lipopolysaccharide from *Salmonella typhimurium* (Sigma) was purified by gel filtration on a Sepharose 4B column using 0.05M ammonium formate, pH 5.5, as the buffer. The first peak eluting off the column was positive for 3-deoxy-D-*manno*-octulosonic acid (KDO) (136) and was pooled, dialyzed against distilled H_2O and lyophilized. *Rhizobium meliloti* 2011 was grown in modified Bergensen's medium (pH 6.9) and harvested in late exponential phase. Cells were harvested by centrifugation at 8,000Xg for 15 min. The cell pellet was vigorously stirred for 12 hrs with water:chloroform:methanol:butanol (4:2:1:1) mixture at room

temperature. After centrifugation, the aqueous phase was removed and extracted sequentially with chloroform and butanol. The aqueous phase (containing LPS) was treated with a solution of DNase I, RNase A, and $MgCl_2$ to remove contaminating nucleic acids. *R. meliloti* 2011 LPS was further purified by gel filtration chromatography on a Sepharose 4B column as described above. Column fractions were monitored for total carbohydrate content by the phenol-sulfuric acid assay (137) and 3-deoxy-D-*manno*-2-octulosonic acid (KDO) by the periodate/thiobarbituric acid assay (136). The LPS was located in the carbohydrate peak that contained large amounts of KDO. LPS was dialyzed against distilled water, concentrated by rotary evaporation, and lyophilized. *Rhizobium trifolii* ANU843 was grown in modified Bergensen's medium (pH 6.9) and harvested at late exponential phase as described above. *R. trifolii* ANU843 LPS was extracted by aqueous phenol as previously described (140). The resulting LPS solution was dialyzed extensively against distilled H_2O, treated with DNase I and RNase A, and purified by gel filtration chromatography as described above.

Lipids and Reagents. 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) was obtained from Molecular Probes, Inc. (Eugene, OR) and was dissolved in 100% ethanol at a concentration of 0.05 mg/ml. Phosphate buffered saline (10mM phosphate, 0.15M NaCl, pH 7.0) containing 0.9mM $CaCl_2$, 2.7mM KCl and 0.5mM $MgCl_2$ (Dulbecco's PBS, D-PBS) was prepared. L-α-phosphatidylcholine (PC), type XVI-E from fresh egg yolk, 99%, was purchased from Sigma.

Fluorescence. Samples for fluorescence measurements were prepared by drying 0.125 mg phosphatidylcholine (PC) and DPH-PC in a vial. DPH-PC was used at a 500:1

PC:DPH-PC molar ratio. The lipids were hydrated in water and the appropriate amount of LPS (in water) added. The samples were then sonicated for 5 minutes with a bath type sonicator, frozen and lyophilized to dryness. Samples were rehydrated in 0.5 ml Dubecco's PBS (D-PBS), heated to 60°C and vortexed. The samples were then frozen, reheated to 60°C and vortexed. This freeze-heat-vortex cycle was repeated several times. Large unilamellar vesicles (LUVs) were prepared by passing the samples 19 times through a liposome extruder equipped with a 100 nm filter (Avisten, Inc., Ottawa, ON). The LUV preparation was diluted 5 fold with D-PBS to a final volume of 2 ml. Fluorescence measurements were made on a SLM 4000 spectrofluorometer (Urbana, IL) with detectors in a T-format and an excitation frequency of 381 nm. A Schott 418 kV bandpass filter was used to remove scattered excitation light. The anisotropy of the DPH-PC probe was calculated according to the following:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where r is anisotropy, and $I_{\parallel}$ and $I_{\perp}$ are the intensity of the parallel and perpendicular fluorescence emission with the excitation light polarized in the parallel orientation. All anisotropy values were corrected for scattered light by using an identical sample without DPH-PC added. The temperature was controlled with a circulating water bath and a jacketed cell. The temperature was measured using a Fluke digital thermometer and a thermocouple placed directly in the sample. All measurements were made at 37.0 ± 0.2°C.

Nuclear Magnetic Resonance Spectroscopy. Samples for NMR analysis were prepared by adding PC (1 mg) in ethanol and drying the solution under a nitrogen stream. The PC was hydrated in 0.6 ml D-PBS and LPS (in water) added. The sample was

sonicated thoroughly with a bath type sonicator, vortexed extensively, frozen and lyophilized to dryness. The sample was taken up into 0.6 ml D_2O and subjected to three freeze-heat-vortexed cycles. The sample was then transferred to a NMR tube and sealed under nitrogen. ^{1}H-NMR spectra were recorded on a Varian 300 MHz spectrometer. Spin-lattice (T1) relaxation times were measured by the inversion recovery technique (169) using a delay-180°-t-90°-acquire pulse sequence where the time, t, was varied and the delay was $\geq$ 4 seconds.

X-Ray Diffraction. X-ray diffraction patterns were recorded on a Rigaku rotaflex rotating anode operating at 45 kV and 100 mA. The raw x-ray beam was collimated with 1/6° slits and the copper K_α line (1.54 Å) was selected by a monochromator. Samples for x-ray analysis were prepared by drying PC, hydrating it into H_2O, and adding *S. typhimurium* LPS. The PC-LPS mixture was sonicated several minutes, frozen, and lyophilized to dryness. The lipids were rehydrated in D-PBS ($\geq$ 60% D-PBS by weight) and heated briefly to 60°C to facilitate hydration. Samples were placed into 0.7 mm glass capillaries (Charles Supper Co.) and flame sealed. Capillaries were mounted in the x-ray beam with a home-built capillary holder. Diffraction patterns were obtained in the transmittance mode. All diffraction patterns were obtained at room temperature (22°C). A one-dimensional scanning detector was used to measure the diffracted radiation.

Results and Discussion

Fluorescence Depolarization Analysis of PC-LPS Membranes. A series of investigations by Jacobs and co-workers (121-124) used fluorescence depolarization to examine the effects of LPS on membranes. They found that LPS caused a small decrease

in the 'fluidity' of the membranes. To date, these studies may be the most thorough and comprehensive examination of the physical properties of phospholipid-LPS mixtures. However, phospholipids in small unilamellar vesicles and diphenylhexatriene (DPH) as the fluorescent lipid probe were employed in these studies. Small unilamellar vesicles are severely strained due to their large curvature and may not exhibit the same properties as non-strained membranes (170). Furthermore, DPH partitions into several regions throughout the acyl chain region of lipid bilayers (171), thereby making the interpretation of the results less clear.

I circumvented these issues by using large unilamellar vesicles and DPH that is covalently linked to PC and is held in a defined position in the membrane. In addition, we measured the effects of *R. trifolii* ANU843 LPS and *R. meliloti* 2011 LPS (as well as *S. typhimurium* LPS) on phosphatidylcholine membranes. The unique structures and biological activities of the LPS from *R. trifolii* and *R. meliloti* allow us to examine the possible correlation between the biological activity and the change in acyl chain dynamics induced by these molecules. The change in the anisotropy of the fluorescent probe as a function of LPS concentration is shown in Figure 3.1. In this experiment, an increase in the anisotropy of the fluorescent probe indicates an increase in the rigidity of the membrane in the environment around the probe. Since the probe is anchored to PC and is located in the fatty acid region of the bilayer, the motion sensed by it is primarily due to the acyl chain mobility of the lipids. All LPS tested increased the anisotropy indicating that they all decreased the mobility of the acyl chains of membranes in which they are incorporated. *R. meliloti* LPS was the most effective at reducing the acyl chain motion while *R. trifolii* and *S. typhimurium* LPS had almost equivalent effects. As seen

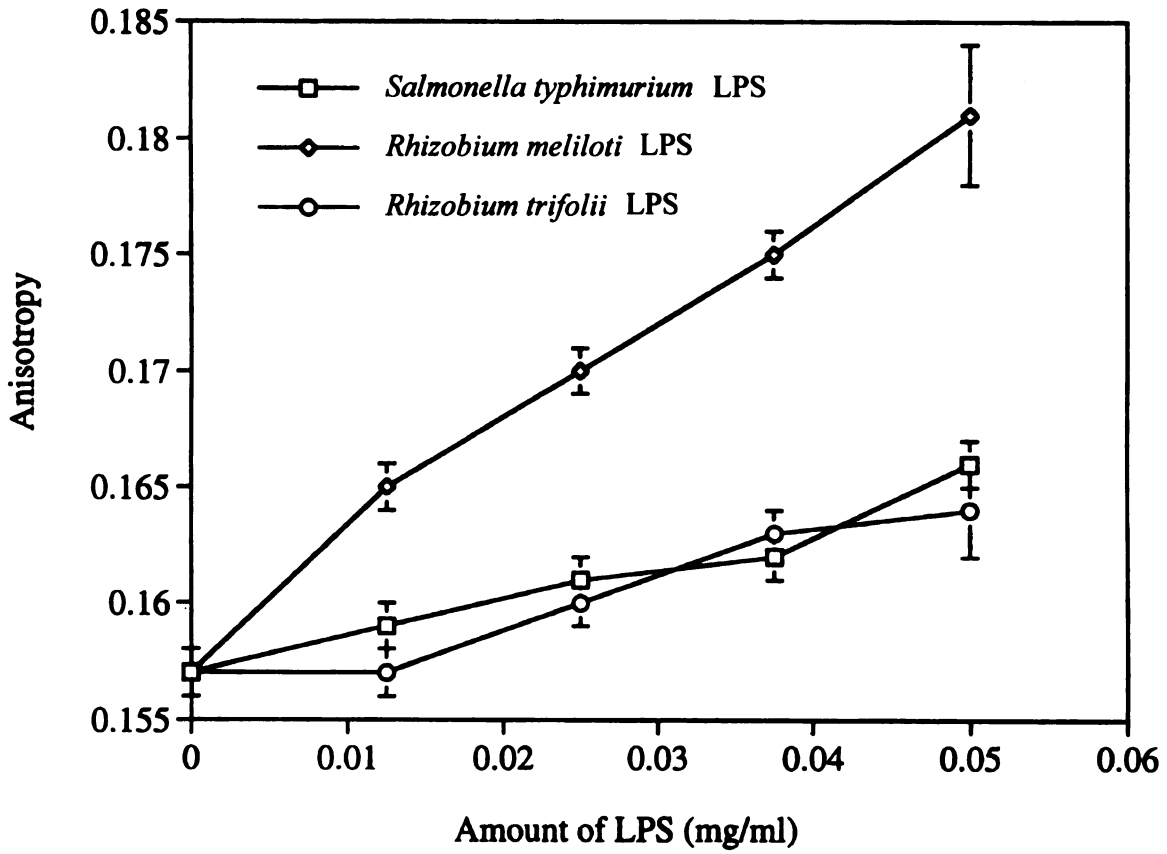

Figure 3.1: Fluorescence anisotropy of DPH-PC in phophatidylcholine membranes (0.05 mg/ml) as a function of LPS concentration.

from the results in Chapter 2, *R. trifolii* and *R. meliloti* LPS have a similar level and spectrum of biological activities. *S. typhimurium* LPS, on the other hand, is much more toxic and pyrogenic and is more effective at eliciting the production of TNF than the rhizobial LPS. These results demonstrate that there is not a simple linear relationship between the acyl chain mobility change induced by LPS (as judged by fluorescence anisotropy) and its biological activity. This is similar to the results of Yeh and Price (122).

The fluorescence depolarization measurements demonstrated the need for a more careful and detailed analysis of the effects of LPS on membranes. To accomplish this, we used x-ray powder diffraction and proton NMR spectroscopy.

X-Ray Diffraction Analysis of PC and PC-S. typhimurium LPS Membranes. X-ray diffraction is a very powerful tool for studying the organization of lipids. In x-ray diffraction, only regular repeating planes of atoms (lattices) diffract the radiation. The angle of the reflection (diffracted x-rays) is governed by Bragg's Law:

$$n\lambda = 2d\sin\theta \qquad (1)$$

where n is the order of the reflection, λ is the wavelength of x-radiation, d is the spacing of the repeating unit, and θ is the angle of the incident and reflected radiation. Lipids typically have two types of repeating structures. The first is due to the bilayer repeat (large d) and gives low-angle reflections (small θ). The second is due to the packing of the acyl chains of the lipids (small d) and gives rise to wide-angle reflections (large θ). The wide-angle region is useful in determining the order of the acyl chains. Acyl chains which are rigid (all-*trans* conformation), such as found in gel phases, give sharp wide-angle reflections. The other extreme is found in acyl chains that have many *gauche*

conformations and have a large degree of motional freedom (fluid-like phases). Acyl chains in this state give broad wide-angle reflections.

The diffraction pattern of phosphatidylcholine (PC) in PBS at room temperature is shown in Figure 3.2. Three equally spaced low-angle reflections (2θ = 1.42°, 2.82°, and 4.36°) are indicative of a lamellar phase with a lamellar repeating distance (d) of 62.2 Å. The wide-angle (acyl chain) reflections of PC were broad and show that the acyl chains were not highly ordered. Therefore, the PC membranes were in the fluid-like, lamellar liquid crystalline phase. Addition of *S. typhimurium* LPS (33% by weight) did not alter the phase of the PC membranes (Figure 3.3). Three discernible lamellar reflections were observed with a similar lamellar repeating distance (64.0 Å) as PC membranes. The PC-LPS diffraction pattern also had broad wide-angle reflections thereby showing that the membranes were in the lamellar liquid crystalline phase.

Upon closer inspection of the wide-angle region, it is obvious that the addition of LPS resulted in significant changes in the acyl chain order of the PC membranes (Figure 3.4). The wide-angle region of both PC and PC-LPS consisted of two overlapping reflections and indicated that the lipids had two acyl chain packing distances. The wide-angle region was deconvoluted into its component reflections (using Lorentzian functions) to aid in its analysis. The parameters of the deconvoluted reflections are given in Table 3.1. Addition of *S. typhimurium* LPS to PC did not induce any significant change to the 3.24 Å reflection. However, LPS caused a dramatic decrease in the intensity (area) of the 4.47 Å acyl chain reflection. It also shifted the 4.47 Å reflection to a smaller repeating distance (4.30 Å) thereby demonstrating that LPS decreased the acyl

Figure 3.2: X-ray diffraction pattern of phosphatidylcholine in D-PBS.
Arrows indicate low-angle reflections.

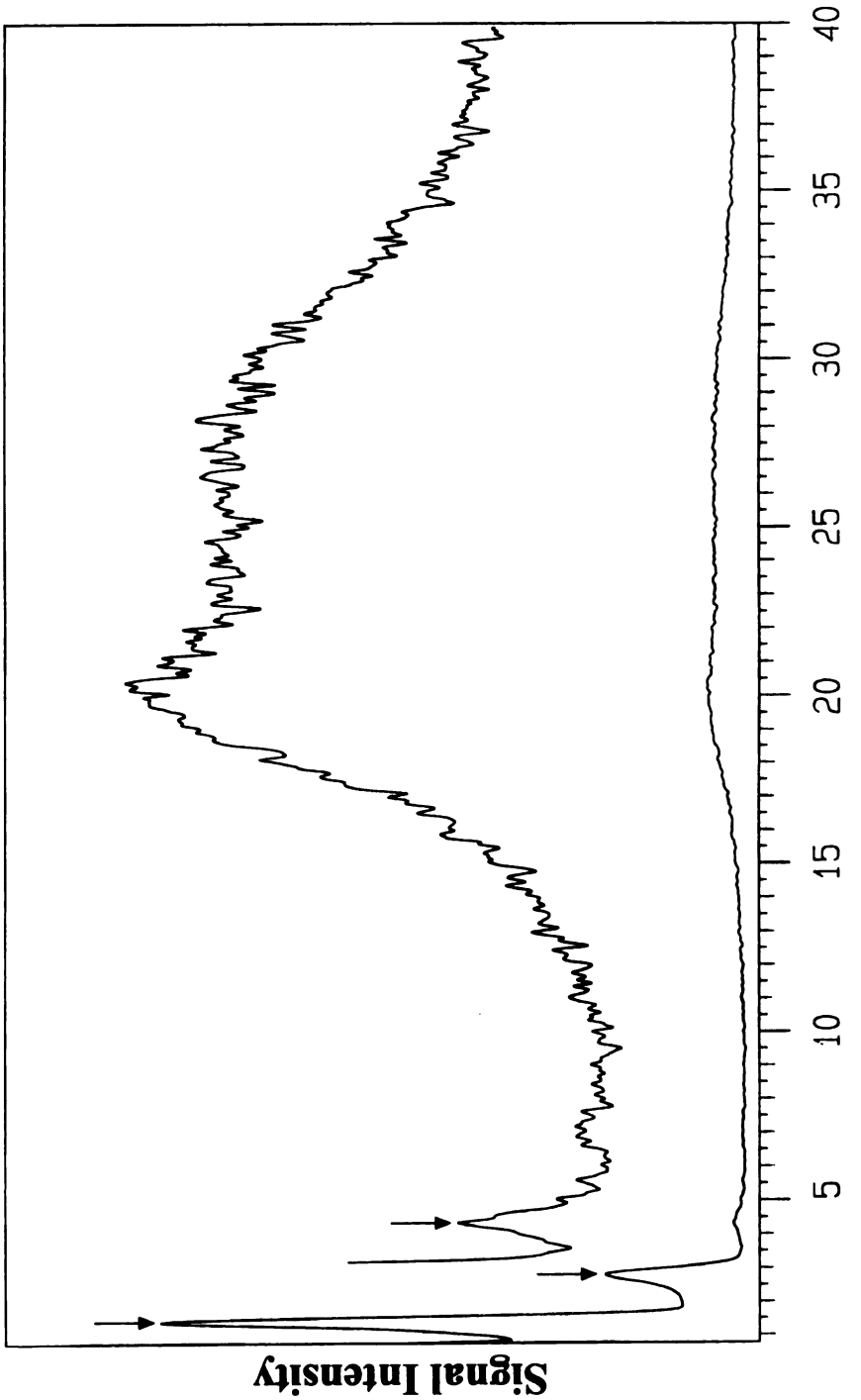

Figure 3.2

Figure 3.3: X-ray diffraction pattern of phosphatidylcholine (67%) – *S. typhimurium* LPS (33%) in D-PBS. Arrows indicate low-angle reflections.

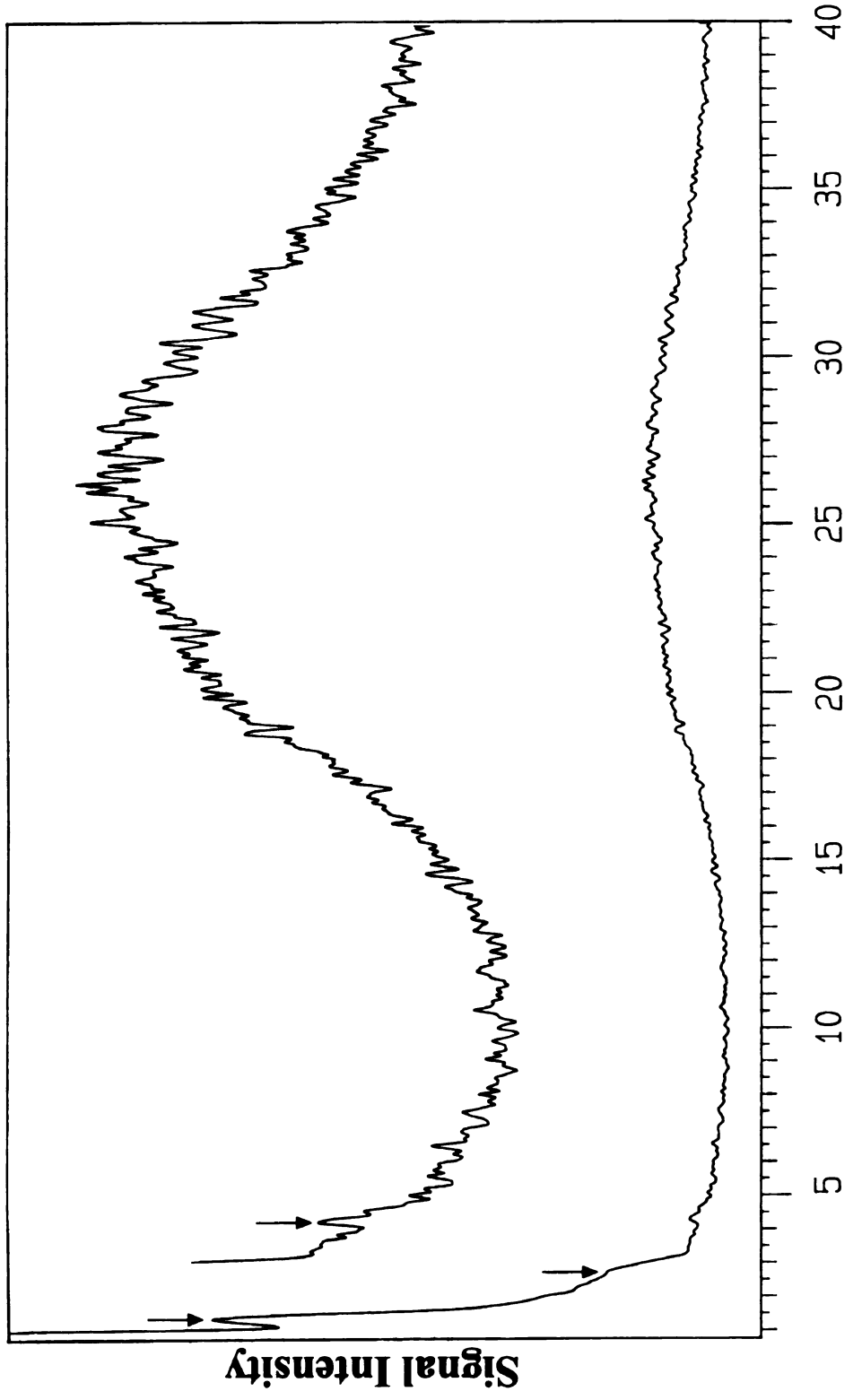

Figure 3.3

Figure 3.4: Wide-angle region of the x-ray diffraction patterns from phosphatidylcholine (A) and 2:1 phosphatidylcholine:*S. typhimurium* LPS (B). Solid lines are the deconvoluted signals and the sum of the deconvoluted signals.

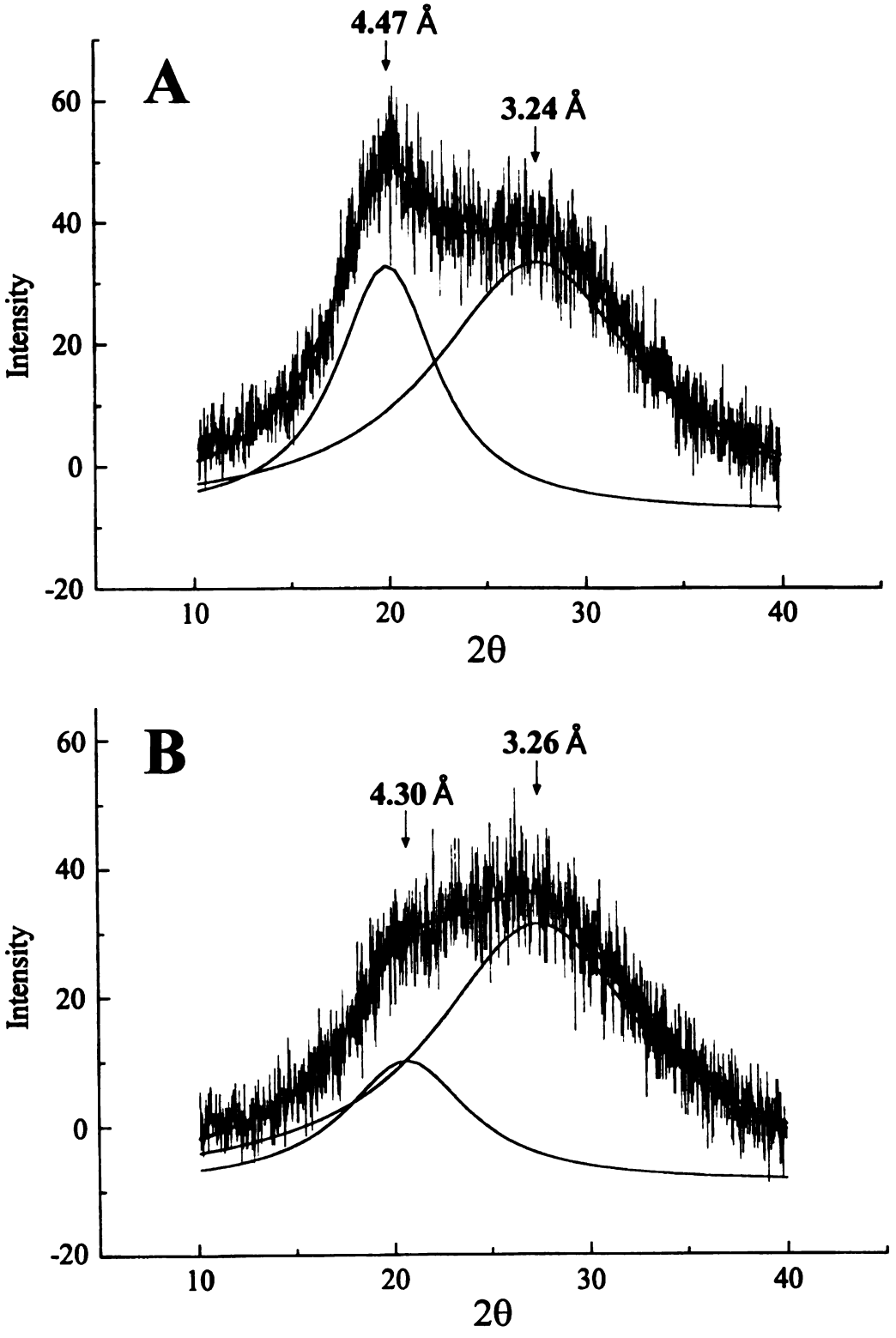
A
4.47 Å
3.24 Å
Intensity
60
40
20
0
-20
10
20
30
40
2θ
B
3.26 Å
4.30 Å
Intensity
60
40
20
0
-20
10
20
30
40
2θ

Figure 3.4

Table 3.1: Parameters of the deconvoluted wide-angle reflections.

	Reflection 1			Reflection 2		
Sample[1]	d (Å)	Peak Width[2]	Area	d (Å)	Peak Width[2]	Area
PC	4.47±0.01	6.2±0.2	394±17	3.24±0.01	12.7±0.3	825±22
PC-LPS[3]	4.30±0.04	7.8±0.5	232±30	3.26±0.03	12.9±0.3	816±36

d=repeat distance
[1]samples contain ≥ 60% Dulbecco's PBS
[2]peak width is given in 2θ degrees
[3]PC-LPS sample contains a 67% PC/33% *S. typhimurium* LPS by weight (before D-PBS is added)

chain packing distance of the membrane. This shows that LPS is increasing the order of the acyl chain. This correlates well with our fluorescence depolarization data as well as previous reports (121, 123, 124, 160-165) which indicated an increase in the order of membranes when LPS is added.

NMR Spectroscopy Analysis of S. typhimurium LPS. The NMR spectrum of pure *S. typhimurium* LPS is shown in Figure 3.5. The very intense signal at 4.7 ppm is the H-O-D resonance. The remaining signals between 3.5-5.5 ppm are primarily due to the protons on the polysaccharide region of LPS. The methylene protons on the acyl chains are observed at 1.0-1.4 ppm. The signal for the methyl protons of the acyl chains, which is normally observed at 0.9 ppm for lipids, is absent. This is the result of severe line broadening due to dipolar coupling and chemical shift anisotropy which occurs for slowly moving nuclei (172) and demonstrates that the methyl groups are severely restricted in their motion.

Of special interest are the signals arising from the methylene protons on the acyl chains (1.0-1.4 ppm). Normally, for lipids in water, the methylene protons give rise to

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one broad signal at approximately 1.3 ppm. These broad signals are due to the overlapping resonances of most of the methylene protons in the acyl chain. Interestingly, the methylene protons for *S. typhimurium* LPS are split into two broad envelopes at 1.22 and 1.34 ppm (Figure 3.5, A). These two signals are due to the protons existing in two or more different orientations (domains) relative to the magnetic field of the NMR spectrometer. The chemical shifts of nuclei in NMR spectroscopy are dependent on the local magnetic field around the nuclei. The local magnetic field shields the nuclei to the applied magnetic field to differing extents and gives rise to changes in the observed chemical shift. The local field is not necessarily uniform in all directions and can be described by the chemical shift tensor, σ:

$$\sigma = \sigma_{11}\lambda_{11}^2 + \sigma_{22}\lambda_{22}^2 + \sigma_{33}\lambda_{33}^2 \qquad (2)$$

where σ_{11}, σ_{22}, and σ_{33} are the principle values of the chemical shift tensor which describe the magnitude in the three Cartesian coordinates and the λ's are the direction cosines which describe the orientation of the molecule with respect to the applied field (172). In 'normal' solution NMR, the molecules are moving fast and, therefore, σ is averaged over all orientations. In rigid poly-crystalline samples, such as liquid crystals (lipids) and polymers, the molecular motion of the sample is slow and the chemical shift depends on the orientation of the sample with respect to the magnetic field. Therefore, the two signals arising from the methylene protons in *S. typhimurium* LPS must be due to at least two different molecular orientations (domains) of the LPS. Interestingly, essentially the same proton spectrum is obtained at any temperature tested (20-70°C), demonstrating the high stability of the packing arrangement of LPS over a broad temperature range (data not shown). The packing was perturbed, however, upon the

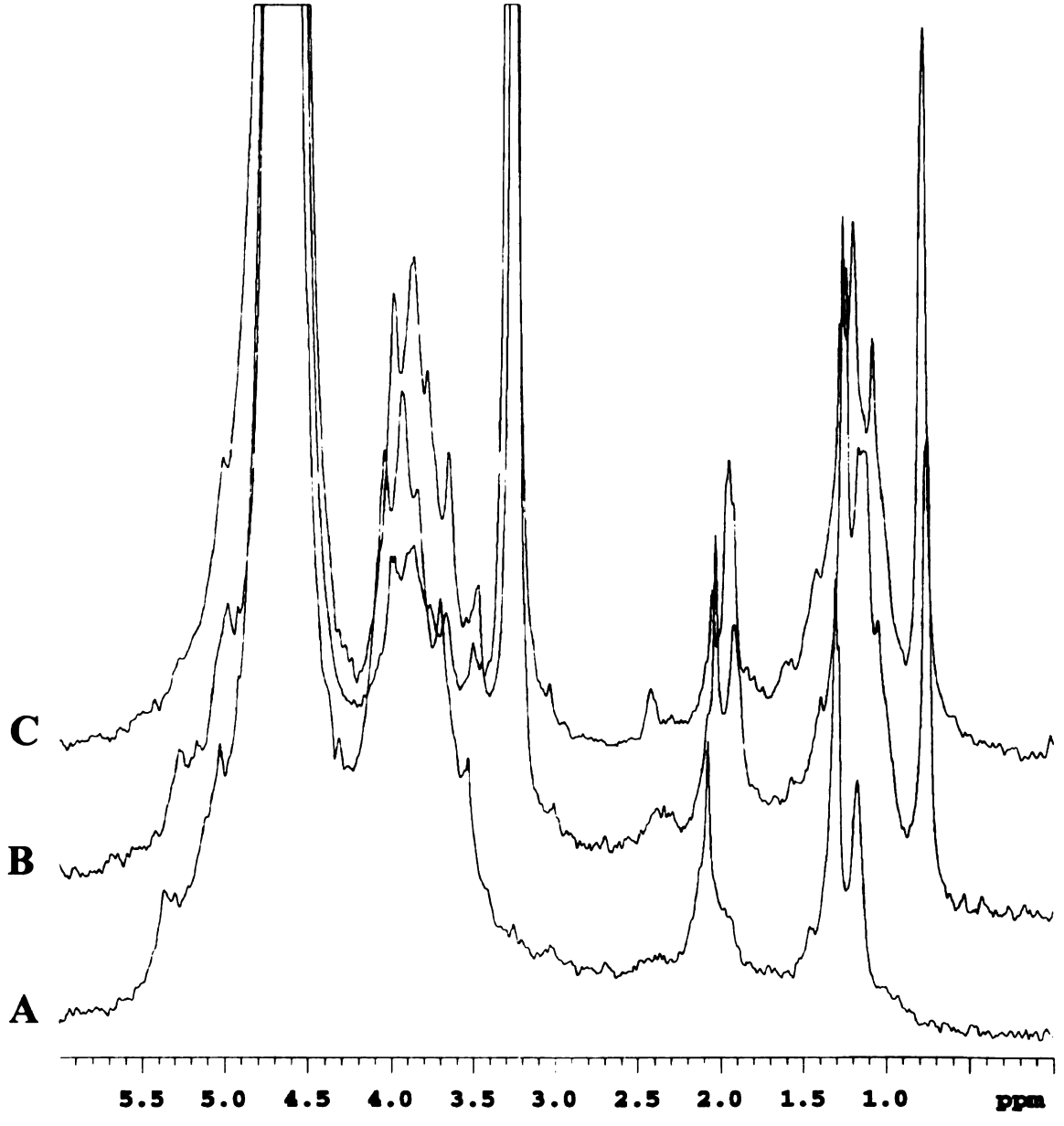

Figure 3.5: ^{1}H-NMR spectra of *S. typhimurium* LPS (37°C) in D-PBS with 0% (A), 25% (B), and 50% (C) of deuterated methanol added. Note the large change in the methylene and methyl protons signals (0.8-1.5 ppm) when methanol is added.

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addition of deuterated methanol to the sample (Figure 3.5, B-C). Addition of deuterated methanol (37°C) completely altered the methylene signals. As the concentration of methanol increased, the methylene signals began to merge into one broad resonance. The methanol also dramatically increased the terminal methyl proton signal (0.9 ppm) indicating that it decreased the rigidity of these protons.

NMR Spectroscopy and T1 Analysis of PC-S. typhimurium LPS Membranes. In order to further examine the effects of *S. typhimurium* LPS on the properties of membranes, we analyzed PC and PC-LPS vesicles by proton NMR spectroscopy. The resultant NMR spectra are shown in Figure 3.6. The NMR spectrum of pure PC (Figure 3.6, A) showed only one methylene signal at 1.29 ppm. As *S. typhimurium* LPS was added (Figure 3.6, B-E), we observe a shift of the methylene signal to 1.34 ppm and the emergence of a second methylene signal at 1.22 ppm. This indicates the presence of at least two different packing states in these lipid mixtures, analogous to the spectrum of pure LPS (Figure 3.5, A). The two methylene signals can not be explained by the simple coexistence of non-interacting LPS and PC membranes. If these two lipids simply coexisted, and assuming that the PC methylene proton signals are at 1.34 ppm, we would expect the ratio of the areas of the 1.34 ppm/1.22 ppm signals to be approximately 13.4/1 at the highest concentration of LPS (Figure 3.6, E). Instead we observe a ratio of 1.2/1. This indicated that a large portion of the 1.22 ppm signal, as well as the 1.34 ppm signal, is due to protons on the acyl chains of the PC. The splitting of the methylene signal, therefore, clearly demonstrates the ability of LPS to induce a dramatic change in the domain structure of PC membranes.

Proton NMR spin-lattice relaxation (T1) times of the methylene protons were

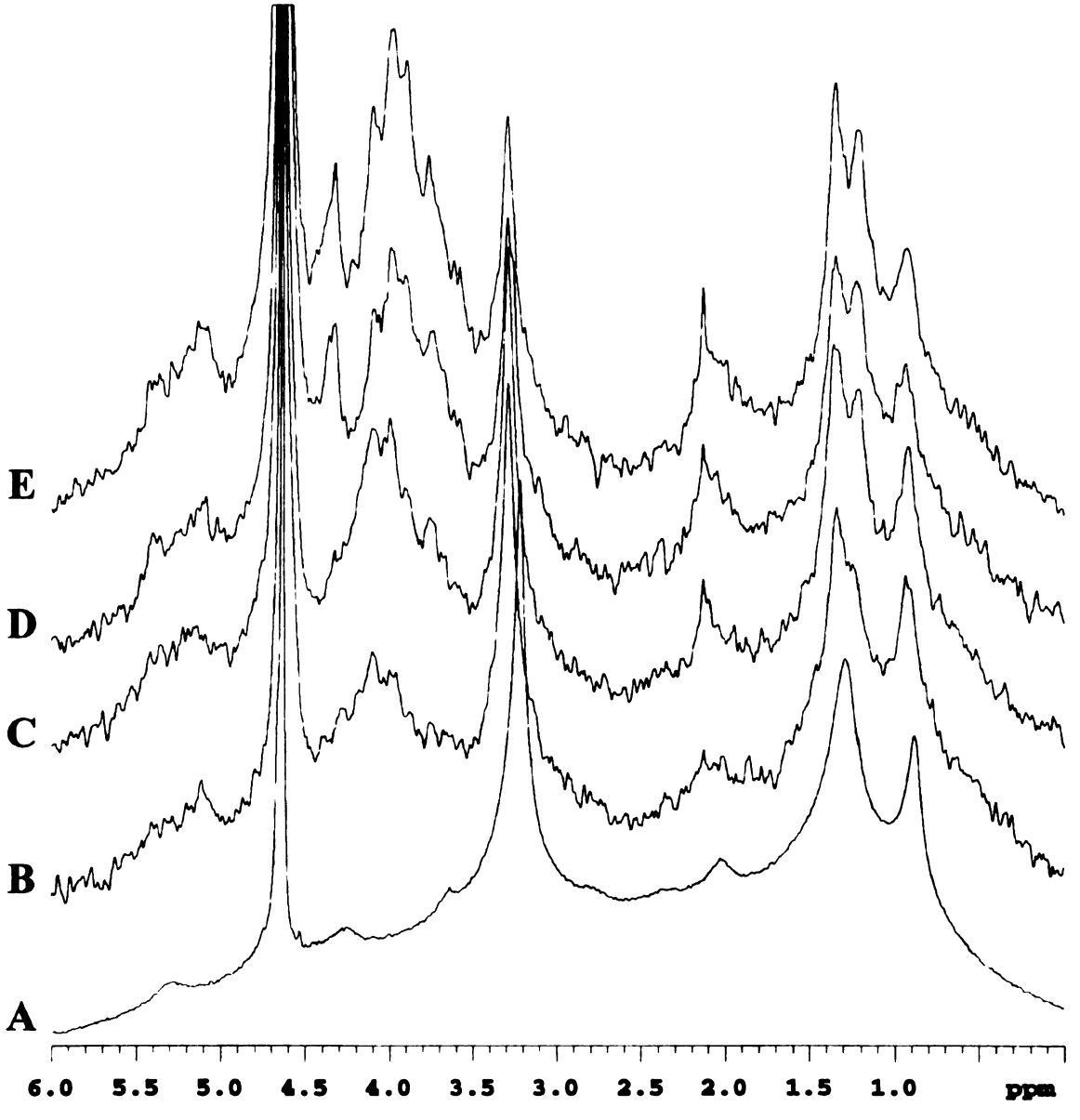


Figure 3.6: ^{1}H-NMR spectra of phosphatidylcholine in D-PBS (37°C) with 0% (A), 20% (B), 33% (C), 43% (D) and 50% (E) *S. typhimurium* LPS (by weight) added. Note the splitting of the methylene proton signal (1.29 ppm) as LPS is added.

analyzed in order to investigate the change in acyl chain dynamics induced by *S. typhimurium* LPS. The NMR T1 experiments were performed using the inversion recovery method (169). In these experiments, the magnetization is inverted (180° pulse) and allowed to recover for variable lengths of time, t. The magnetization that recovers along the z-axis (parallel to the magnetic field) is then rotated 90° into the x-y plane and measured. The recovery of the magnetization to equilibrium is exponential and is described by the equation:

$$M_\infty - M_t = 2M_\infty \exp(-t/T1) \qquad (3)$$

where M_∞ and M_t are the magnetization at equilibrium and at time = t, t is the delay time and T1 is the spin-lattice relaxation time (172). For spin 1/2 nuclei, dipole-dipole interaction is the primary mechanism for relaxation (i.e. the interaction of a proton with another proton). T1 relaxation is dominated by the relaxation events occurring near the same rate as the NMR spectrometer frequency. For lipids at 300 MHz ($\sim 10^{-9}$ s), this corresponds to the *trans-gauche* isomerization of the carbon-carbon bonds in the acyl chains. Kroon et al. (173) have demonstrated that the T1 relaxation of lipid acyl chains is due to the intra-chain proton-proton interaction resulting from the *trans-gauche* rearrangement. Hence, the T1 relaxation times are measuring the rate of *trans-gauche* isomerization of the lipid acyl chains. For our experiments, the T1 relaxation is in the fast correlation region where an increase in *trans-gauche* isomerization rate results in an increase in T1.

Rearranging equation 3 and substituting the magnetization with the intensity of the NMR signal (I) we obtain:

$$\ln(I_\infty - I_t) = \ln(2I_\infty) + -t/T1. \qquad (4)$$

For a single exponential recovery, a plot of $\ln(I_\infty - I_t)$ vs. t will give a straight line with a slope equal to -1/T1. Plots of $\ln(I_\infty - I_t)$ vs. t for the methylene protons of PC and LPS-PC membranes are shown in Figure 3.7. The one methylene signal observed for pure PC membranes gives a straight line plot indicating that it is a single exponential recovery with T1 = 0.54 seconds. None of the samples that contained LPS gave a straight line and are, therefore, not adequately described by a single exponential recovery. The data for these samples were fit to a double exponential equation:

$$I_\infty - I_t = C_a\exp(-t/T1_a) + C_b\exp(-t/T1_b)] \qquad (5)$$

where C_a and C_b are variable terms used to describe the $2I_\infty$ term in equation 4. All of the inversion recovery plots for the methylene signals were fit very well by this equation (correlation coefficients ≥ 0.999). The T1 values obtained by this analysis are given in Table 3.2. The values of $T1_a$ for the 1.34 ppm peak did not change much from pure PC membranes demonstrating that LPS had little influence on the *trans-gauche* isomerization rate of these protons. At low concentrations of LPS, the $T1_a$ values for the 1.22 ppm signal were very similar to that of the 1.34 ppm signal indicating that both of these domains had a similar rate of *trans-gauche* isomerization in their acyl chains. At high LPS concentrations, $T1_a$ for the 1.22 ppm signal began to decrease. Thus, the rate of *trans-gauche* isomerization for the protons in this signal is decreased by high levels of LPS. From the data it can be seen that the second exponential term (which gives $T1_b$) was highly variable. This is due in large part to the fact that this exponential term is most strongly influenced by the data at long recovery times where small errors in the intensity of the NMR signal will result in a large fluctuations in the T1 value. While we know that the second exponential term is due to slowly relaxing protons (fast motion), the

Figure 3.7: ^{1}H-NMR T1 recovery plots for the methylene protons of PC and PC-*S. typhimurium* LPS membranes at 37°C. The straight lines are approximate fits to the early time points and are used to illustrate the non-linear behavior of the data when LPS is added.

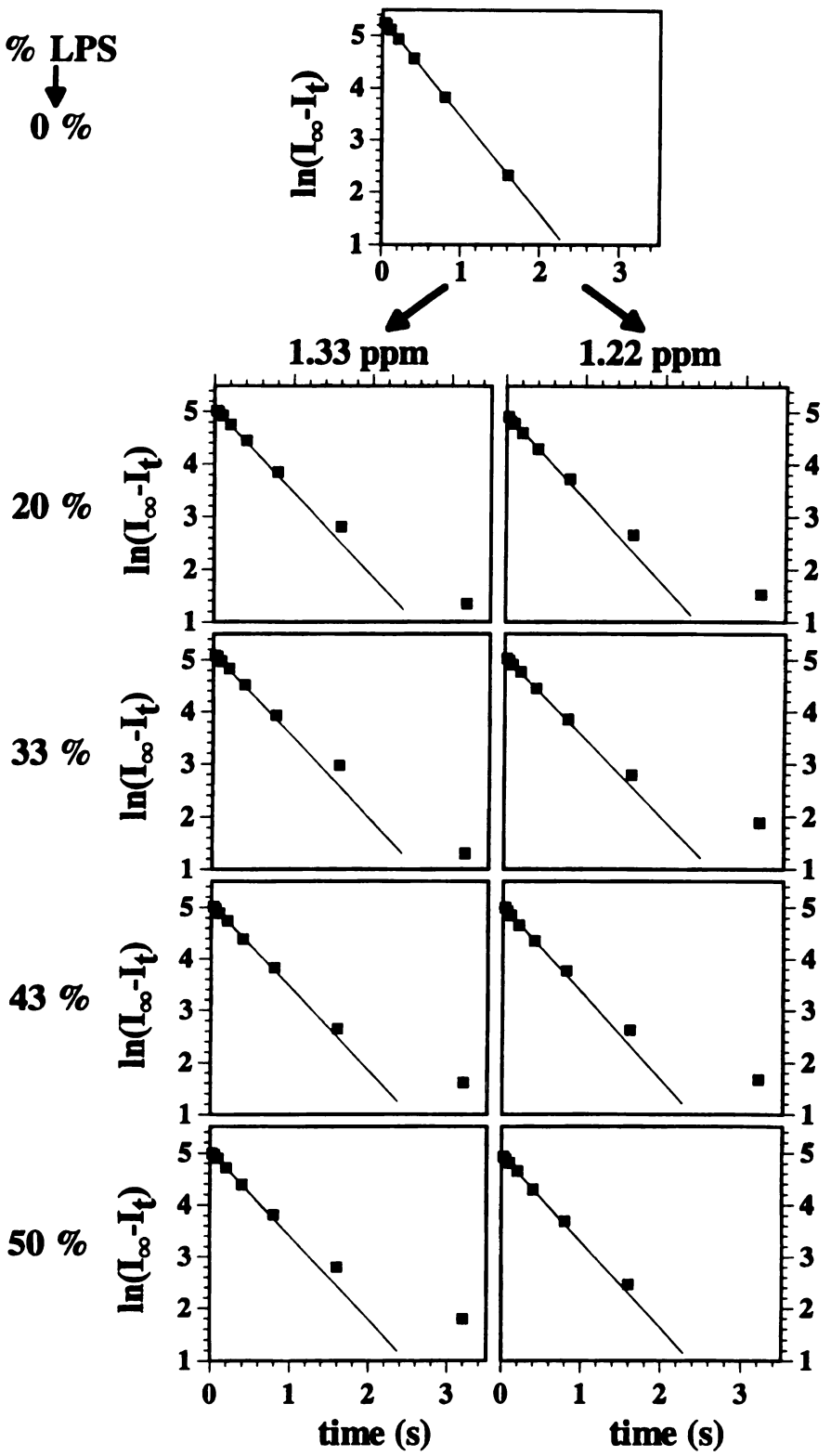


Figure 3.7

Table 3.2: T1 values for the methylene protons in PC and LPS-PC membranes.

S. typhimurium LPS (% PC by weight)	1.34 ppm signal		1.22 ppm signal	
	$T1_a$ (s)	$T1_b$ (s)	$T1_a$ (s)	$T1_b$ (s)
0%	0.54[1]	na	na	na
20%	0.61	3.34	0.54	2.03
33%	0.56	1.47	0.64	-2.48
43%	0.58	9.43	0.49	1.71
50%	0.56	3.80	0.38	0.67

na=not applicable

[1] 1.29 ppm

variability of the $T1_b$ values precludes a quantitative examination of this term.

As discussed above, both the 1.34 ppm and 1.22 ppm signals are comprised of methylene protons from LPS and PC. Therefore, it is impossible to determine unequivocally whether or not each of the exponential recoveries arise from one of the lipid species or from a mixed aggregate that has the same motional properties. It is clear, however, that each of the two methylene signals observed for mixed LPS-PC membranes contains protons in at least two different motional states.

To obtain a clear understanding of the effects that LPS has on membranes, it is instructive to compare the fluorescence depolarization and NMR T1 measurements. An excellent study by Petersen and Chan (11) showed that two types of motions could best describe acyl chain dynamics. The first is the large motions of the acyl chains induced by a single *gauche* conformation (chain reorientation) and occurs at approximately 10^{-7} s. The second is from the coupled *trans-gauche* isomerizations that give rise to kinks in the acyl chains. Successive *trans-gauche(+)/gauche(-)-trans* isomerizations result in the

diffusion of the kink along the acyl chain and occur at approximately 10^{-10} s. Since the fluorescence measurements are sensitive to motions occurring at about 10^{-8} s, they would be mainly measuring the chain reorientation motions. Likewise, the NMR T1 measurements (at 300 MHz) are sensitive to motions occurring at about 10^{-9}-10^{-10} s and would be primarily measuring the *trans-gauche* isomerizations in the acyl chains that give rise to kink diffusion. In the NMR results, only one of the methylene signals showed a decrease in T1 in response to LPS and this decrease was relatively small. This indicates that *Salmonella* LPS has a relatively small influence on the rate of kink diffusion in the membrane. The fluorescence experiments, on the other hand, demonstrated that LPS caused a significant decrease in the chain reorientation motions of phosphatidylcholine membranes. Thus it appears that LPS alters membrane dynamics primarily by decreasing the large acyl chain fluctuations. However, this does not account for the sub-population of methylene protons observed in the NMR T1 measurements that had an increased rate of acyl chain kink diffusion. We would expect that these regions of increased motions would decrease the fluorescence anisotropy of the lipid probe, assuming that the probe partitions into this area of the membrane. However, these steady-state fluorescence measurements could not distinguish sub-populations of different acyl chain motions because they are derived from an averaged signal. Therefore, the different populations of motions measured by NMR T1, including the increase in the rate of kink diffusion, will be averaged into the fluorescence anisotropy results.

Conclusions

From the fluorescence depolarization, x-ray diffraction, proton NMR, and proton NMR T1 relaxation measurements, we can see the large influence that LPS has on the dynamics and order of PC membranes. X-ray diffraction measurements clearly showed that LPS (at the concentration and temperature tested) did not alter the phase of PC membranes but did decrease the acyl chain packing distance. The fluorescence depolarization measurements demonstrate that LPS decreases the acyl chain mobility of membranes in which it is incorporated. The NMR T1 measurements also showed a small decrease in the acyl chain mobility induced by LPS. Interestingly, the fluorescence depolarization measurements utilizing rhizobial LPS indicated that there is not a linear relationship between acyl chain mobility and its ability to elicit a physiological response. This suggests that the relationship between membrane physical property changes and physiological responses elicited by LPS may be very complex and likely involves many aspects of membrane dynamics and order.

Analysis of the NMR spectra from PC-LPS mixtures indicates that LPS causes a large change in membrane order. LPS induced the lateral separation of lipids into distinct domains. The two NMR signals observed for these domains had different responses to LPS; the 1.22 ppm protons decreased in motion as the concentration of LPS increased whereas the 1.34 ppm protons did not. Both of these signals, however, were not consistent with a single exponential recovery (as seen for pure PC membranes) and therefore are comprised of protons in at least two different motional states. This included a fast motion component and suggests that LPS induced regions of membrane defects or regions of different domains.

Our results have provided a much more detailed molecular understanding of the membrane perturbations induced by LPS than was previously known. These results definitively demonstrate that *S. typhimurium* LPS causes a dramatic change in both the dynamics and order of membranes in a very complex manner. Furthermore, they lend credence to the hypothesis that membrane perturbations are a critical component of the physiological responses elicited by LPS. Our findings clearly demonstrate that membrane perturbations are a possible, if not probable, mechanism by which LPS activates immune cells.

Since LPS presumably trigger a cellular response through alterations in membrane dynamics and order, it should be expected that other factors that affect membrane properties could affect this response. Environmental conditions such as temperature, ionic strength, pH and pressure will affect membrane properties. Lipophilic molecules such as solvents and lipids will also induce membrane perturbations. If LPS act at the membrane level, then we should expect that environmental conditions would alter its ability to induce a cellular response. Moreover, the membrane perturbations elicited by LPS can be thought of as a stress or shock to the cell, similar to other environmental conditions such as temperature. Interestingly, there is a link between thermal shock in macrophages and their ability to produce cytokines. Several investigators demonstrated that heat shock decreases the production of tumor necrosis factor (TNF) from macrophages (174-176). Cold shock, on the other hand, enhances the production of TNF and interleukins from LPS stimulated macrophages (177, 178). These results indicate that LPS effects and thermal effects may be related. This is consistent with a role of membrane perturbations in the mechanism of LPS action.

Chapter 4

Probing the Mechanism of Lipopolysaccharide Action with Synthetic Lipids

Introduction

Lipopolysaccharides (LPS) are complex glycolipids located on the outer leaflet of the outer membrane of Gram-negative bacteria. LPS are capable of eliciting a wide range of biological activities when they interact with the immune system of animals and are responsible for the deleterious effects of Gram-negative septicemia. These biological activities include the induction of synthesis of tumor necrosis factor (TNF) and interleukins by macrophages, the induction of the proliferation of B-cells, and the activation of the clotting enzyme cascade of the lysate of amebocytes from the horseshoe crab (see Chapter 1).

The critical structural elements of LPS that determine biological activity are found in the lipid A region of the molecule. There is a very fine relationship between lipid A structure and the various biological activities of LPS. The structures of the fatty acids are critical elements of this relationship. For example, the lipid A head group might be completely exposed, as in free lipid A, or covered with hundreds of carbohydrate groups containing substituents such as phosphate groups (smooth form lipopolysaccharide) without really influencing its biological activity (73, 89). However, the insertion of double bonds into fatty acids and modification of fatty acid chain lengths are sufficient to completely abolish endotoxin activity. The lipid A of *Rhodobacter sphaeroides* is identical in structure to that of the Enterobacteriaceae except that some of the fatty acyl chains are shorter and one is unsaturated (95), yet this lipopolysaccharide is completely devoid of endotoxin activity (95-99).

In 1987, Jacobs and Price (121) proposed a 'two-step' model to explain the mechanism by which lipid A interacts with and activates immune cells. This model

attempts to bring together the seemingly distant views of receptor mediated versus membrane mediated events in the activation of immune cells by LPS. This model was based, in large part, on studies that showed that LPS interacted with cells in two distinct steps. First, lipid A (or LPS) binds to the membrane of the host cell. Binding is proposed to involve an electrostatic interaction between lipid A and the cell surface. These authors suggest that a membrane-associated protein (LPS receptor) may mediate this interaction. Next, lipid A inserts (fuses) into the membrane of the immune cell. The insertion of lipid A into the membrane results in the activation of the immune cell.

After LPS inserts into the host cell membrane, it induces a signal that causes the cell to become more active and, for instance, secrete cytokines. While the origin of this signal is still undefined, it may come from LPS-induced perturbations in the properties of the host cell membrane. This contention is supported by our results (Chapter 3) which clearly demonstrate that LPS are capable of dramatically altering the dynamics and order of membranes. The ability of LPS to affect lipid dynamics and order would be dependent on its molecular and aggregate structure. Manipulating LPS in such a way as to change its molecular order or alter its ability to form highly ordered aggregates would alter its effects on the properties of membranes. If membrane perturbations were involved in the mechanism of LPS action, these changes should affect its biological activities. In fact, the biological activities of LPS are highly dependent upon its structural and physicochemical properties (7, 125, 126, 129). Furthermore, we should expect that molecules that alter these properties of LPS or offset the membrane perturbations induced by LPS would drastically alter the biological activities of LPS.

In 1994, our lab reported the synthesis of two positively charged lipids that were designed to interact with the negatively charged LPS (179). These lipids were found to block the ability of LPS to activate the clotting enzyme cascade of the horseshoe crab (*Limulus*) amebocyte lysate (LAL assay) and were named endotoxin inhibitors (EI-1 and EI-2). The mechanism by which EI-1 and EI-2 were able to block the activity of LPS was not determined. However, because these lipids were designed to interact with LPS, it was suggested that they disrupt the high degree of order of LPS aggregates and thus alter the ability of LPS to induce membrane perturbations. Furthermore, the ability of EI-1 and EI-2 to disrupt cells at high concentrations led Lill-Elghanian to suggest that these lipids may act as membrane 'fluidizers' (133).

In this report, I am using EI-1 and EI-2, as well as three additional lipids that were synthesized (EI-3, EI-4 and EI-5), to investigate the mechanism by which LPS activates immune cells (Figure 4.1). These five synthetic lipids can be viewed as molecular probes to manipulate the interaction between LPS and immune cells. By understanding how the five synthetic lipids affect the biological activity of LPS and how they alter the LPS-host cell interaction, we may gain an insight into the mechanism of LPS action. The synthesis, purification, structural characterization, and physical properties of EI-3, EI-4 and EI-5 are described later (Appendix I). Here I describe the effects that these five lipids have on the biological activities of LPS (LAL assay and TNF-α induction from macrophages) and their ability to alter the dynamics and order of LPS-phospholipid membranes.

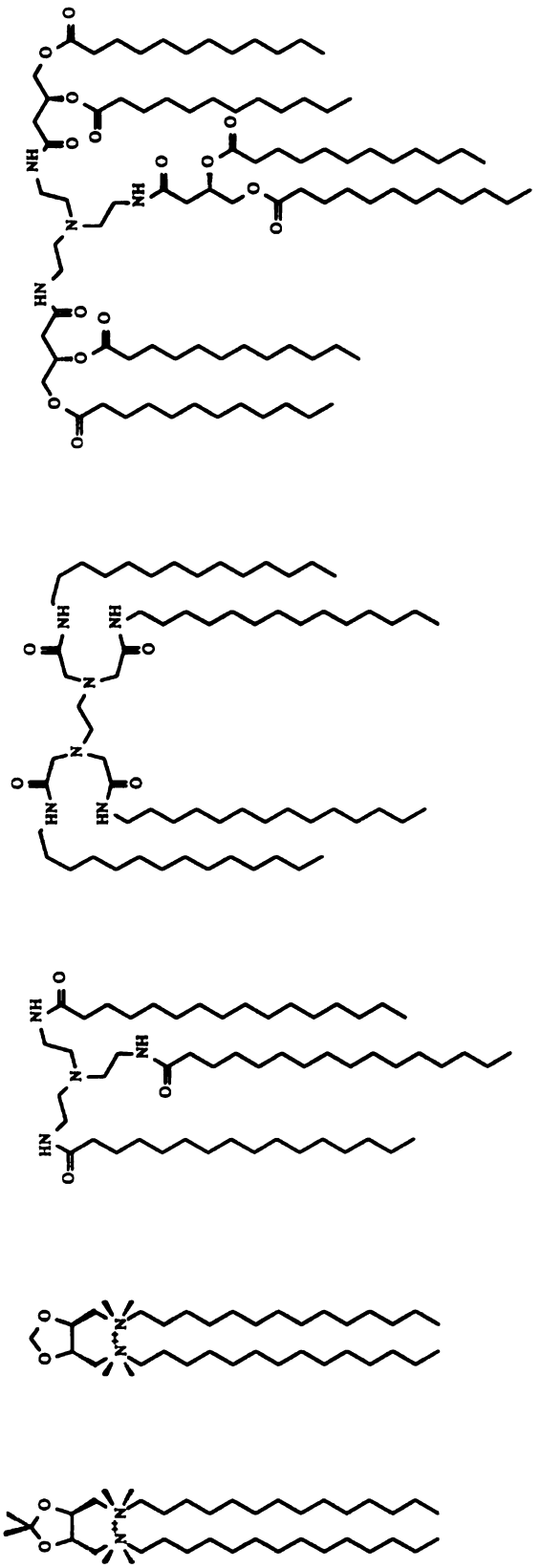


Figure 4.1: Structures of the five synthetic lipids.

Materials and Methods

LPS, Lipids and Reagents. Lipopolysaccharide from *Salmonella typhimurium* (Sigma) was purified by gel filtration chromatography on a Sepharose 4B column using 0.05M ammonium formate, pH 5.5, as the buffer. The first carbohydrate-containing peak (137) eluting off the column was positive for 3-deoxy-D-*manno*-octulosonic acid (KDO) (136) and was pooled, dialyzed against distilled H_2O and lyophilized. EI-1 and EI-2 were prepared previously (179). 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) and *trans*-parinaric acid (tPnA) were obtained from Molecular Probes, Inc. (Eugene, OR) and were dissolved in 100% ethanol at a concentration of 0.05 mg/ml. Phosphate buffered saline (10mM phosphate, 0.15M NaCl, pH 7.0) containing 0.9mM $CaCl_2$, 2.7mM KCl and 0.5mM $MgCl_2$ (Dulbecco's PBS, D-PBS) was prepared. L-α-phosphatidylcholine (PC), type XVI-E from fresh egg yolk, 99%, was purchased from Sigma. Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids, Inc., Alabaster, Alabama.

Fluorescence. Samples for fluorescence measurements of PC-LPS membranes were prepared by drying 0.125 mg phosphatidylcholine (PC), the synthetic lipids, and DPH-PC in a vial. The synthetic lipids were added at the molar ratio of synthetic lipid/LPS indicated, assuming a molecular weight of LPS of 15000 g/mole. DPH-PC was used at a 500:1 PC:DPH-PC molar ratio. The lipids were hydrated in water and 0.125 mg of LPS (in water) added. The samples for DPPC and DPPC-LPS membranes were prepared in the same way except that DPPC was substituted for PC and tPnA was used as the fluorescent membrane probe at a DPPC:tPnA molar ratio of 200:1. All of the samples

were sonicated for 5 minutes with a bath type sonicator, frozen and lyophilized to dryness. Samples were rehydrated in 0.5 ml Dubecco's PBS (D-PBS), heated to 60°C and vortexed. The samples were then frozen, reheated to 60°C and vortexed. This freeze-heat-vortex cycle was repeated several times. Large unilamellar vesicles (LUVs) were prepared by passing the samples 19 times through a liposome extruder equipped with a 100 nm filter (Avisten, Inc., Ottawa, ON). The LUV preparation was diluted 5 fold with D-PBS to a final volume of 2 ml. Fluorescence measurements were made on a SLM 4000 spectrofluorometer (Urbana, IL) with detectors in a T-format and an excitation frequency of 381 nm for DPH-PC or 320 nm for tPnA. A Schott 418 kV bandpass filter was used to remove scattered excitation light. The anisotropy of the DPH-PC probe was calculated according to the following:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where r is anisotropy, and $I_{\parallel}$ and $I_{\perp}$ are the intensity of the parallel and perpendicular fluorescence emission with the excitation light polarized in the parallel orientation. All anisotropy values were corrected for scattered light by using an identical sample without the fluorescent lipid probe added. The temperature was controlled with a circulating water bath and a jacketed cell. The temperature was measured using a Fluke digital thermometer and a thermocouple placed directly in the sample. Sample temperatures were ± 0.2°C from the reported value.

Nuclear Magnetic Resonance Spectroscopy. Samples for NMR analysis were prepared by adding PC (1 mg) and the synthetic lipids and drying the solution under a nitrogen stream. The lipids were hydrated in 0.6 ml D-PBS and LPS (in water) added. The sample was sonicated thoroughly with a bath type sonicator, vortexed extensively,

frozen and lyophilized to dryness. The sample was taken up into 0.6 ml D_2O and subjected to three freeze-heat-vortex cycles. The sample was then transferred to an NMR tube and sealed under nitrogen. ^{1}H-NMR spectra were recorded on a Varian 300 MHz spectrometer. Spin-lattice (T1) relaxation times were measured by the inversion recovery technique (169) using a delay-180°-t-90°-acquire pulse sequence where the time, t, was varied and the delay was $\geq$ 4 seconds.

Cell Lines. RAW264.7, a murine Abelson leukemia virus-transformed lymphocyte with macrophage properties (141), was obtained from American Type Culture Collection. RAW264.7 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 mg/L streptomycin sulfate (culture medium). Subcultures were prepared by scraping and resuspending in fresh culture medium. Cells were grown at 37°C in a humidified, 5% CO_2 atmosphere.

Tumor Necrosis Factor-α Assay. S. typhimurium LPS and the inhibitors (in ethanol) were added to 1 ml of distilled water and sonicated. Samples were lyophilized, rehydrated in Dulbecco's PBS and then sonicated and vortexed thoroughly. RAW264.7 cells (2×10^5) were placed in 2 ml of culture medium in 24 well culture plates and allowed to grow for 15 hrs. Samples (50 µl) were added to the cell culture wells and allowed to incubate with the cells at 37°C, 5% CO_2. Cells were allowed to incubate with the samples for 8 hrs since a time course experiment determined that the maximal TNF response occurred at this time. After incubation, cell culture supernatants were removed, centrifuged to remove cell debris, and placed on ice. The TNF-α concentration in the culture supernatant was determined by using a mouse TNF-α ELISA kit (Genzyme Corp., Cambridge, MA). TNF-α levels were quantitated with recombinant mouse TNF-α

according to the test kit instructions. The results shown are the average of duplicate determinations and the error bars show the range of the results.

Limulus amebocyte lysate (LAL) assay. The LAL assay was performed with the Whittaker Bioproducts QCL-1000 Quantitative Chromogenic LAL, Whittaker Bioproducts, Inc. (Walkersville, MD). All assays were standardized to Endotoxin Units (EU) using *E. coli* 0111:B4 LPS according to the assay instructions. Samples were prepared by adding *S. typhimurium* LPS and the inhibitors (in ethanol) to distilled water. The samples were sonicated and then lyophilized to dryness. The samples were rehydrated in distilled water and sonicated and vortexed thoroughly immediately prior to being assayed. The final concentration of *S. typhimurium* LPS was 0.1 ng/ml. Results shown are the mean of at least three determinations and the error bars indicate the standard deviation.

Results and Discussion

In Chapter 3, I demonstrated that LPS has a dramatic influence on the dynamics and order of membranes. These results are consistent with the idea that LPS-induced membrane perturbations are a critical component of its ability to elicit a severe immune response in animals. However, little direct evidence exists to support the correlation between membrane property changes induced by LPS and its ability to elicit a biological response. The discovery that some positively charged synthetic lipids (EI-1 and EI-2) could block the biological response induced by LPS (179) suggested that these lipids could be used to probe the mechanism of LPS action. Lill-Elghanian proposed that EI-1 and EI-2 might act as membrane 'fluidizers' (133). To examine this possibility further,

we synthesized three additional lipids that all have a different number of acyl chains (Figure 4.1). By increasing the number of acyl chains in a lipid, we will increase the van der Waals interactions within this lipid and thus, increase the molecular order of the lipid. In turn, this will alter its effects on the properties of LPS and membranes.

The effects of the five synthetic lipids (EI-1 through EI-5) on the biological activities of *S. typhimurium* LPS were determined by their ability to reduce LPS activation of the LAL assay and their ability to reduce LPS-dependent production of tumor necrosis factor-α (TNF-α) from a macrophage cell line. The LAL assay (Figure 4.2) showed conclusively that all of the synthetic lipids were able to reduce the activation of the LAL cascade (using 0.1 ng/ml *S. typhimurium* LPS). EI-1 and EI-2 were the most potent inhibitors and completely blocked the LAL cascade at 100 ng/ml. EI-3, EI-4, and EI-5 were less effective inhibitors and at 100 ng/ml gave 85%, 46%, and 50% inhibition, respectively. EI-3, EI-4, and EI-5 were not able to elicit an LAL response by themselves. EI-1 and EI-2 have been shown previously to be inactive in the LAL assay (Hollingsworth and Lill-Elghanian, unpublished data). Using the same concentrations as tested in the LAL assay, the synthetic lipids were also tested for their ability to inhibit the production of TNF-α from RAW264.7 cells induced by 0.1 ng/ml *S. typhimurium* LPS (Figure 4.3). RAW264.7 cells are a macrophage-like cell line known to be activated by LPS (141). All of the synthetic lipids, with the exception of EI-4, were able to completely block the induction of TNF-α at the concentrations tested. The TNF-α induction assay also showed that EI-1 and EI-2 did not alter the basal level of TNF-α produced. The decrease in TNF-α production seen for the LPS treated cells was not due to a nonspecific toxicity of the lipids since the basal level of TNF-α production did not

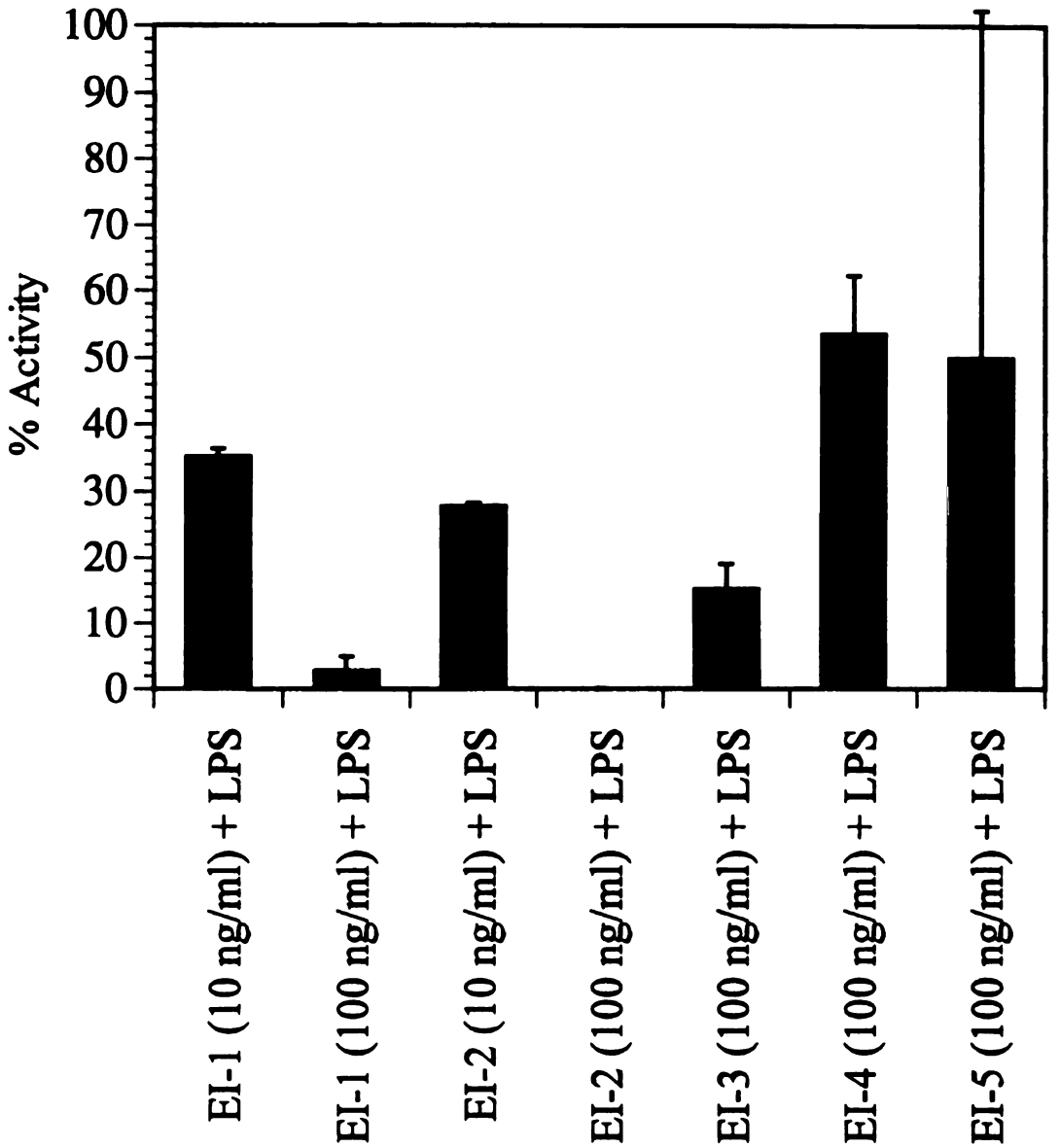


Figure 4.2: Effect of the synthetic lipids on the activity of 0.1 ng/ml *S. typhimurium* LPS in the *Limulus* amebocyte lysate (LAL) assay. All values are reported as the percent activity relative to 0.1 ng/ml *S. typhimurium* LPS.

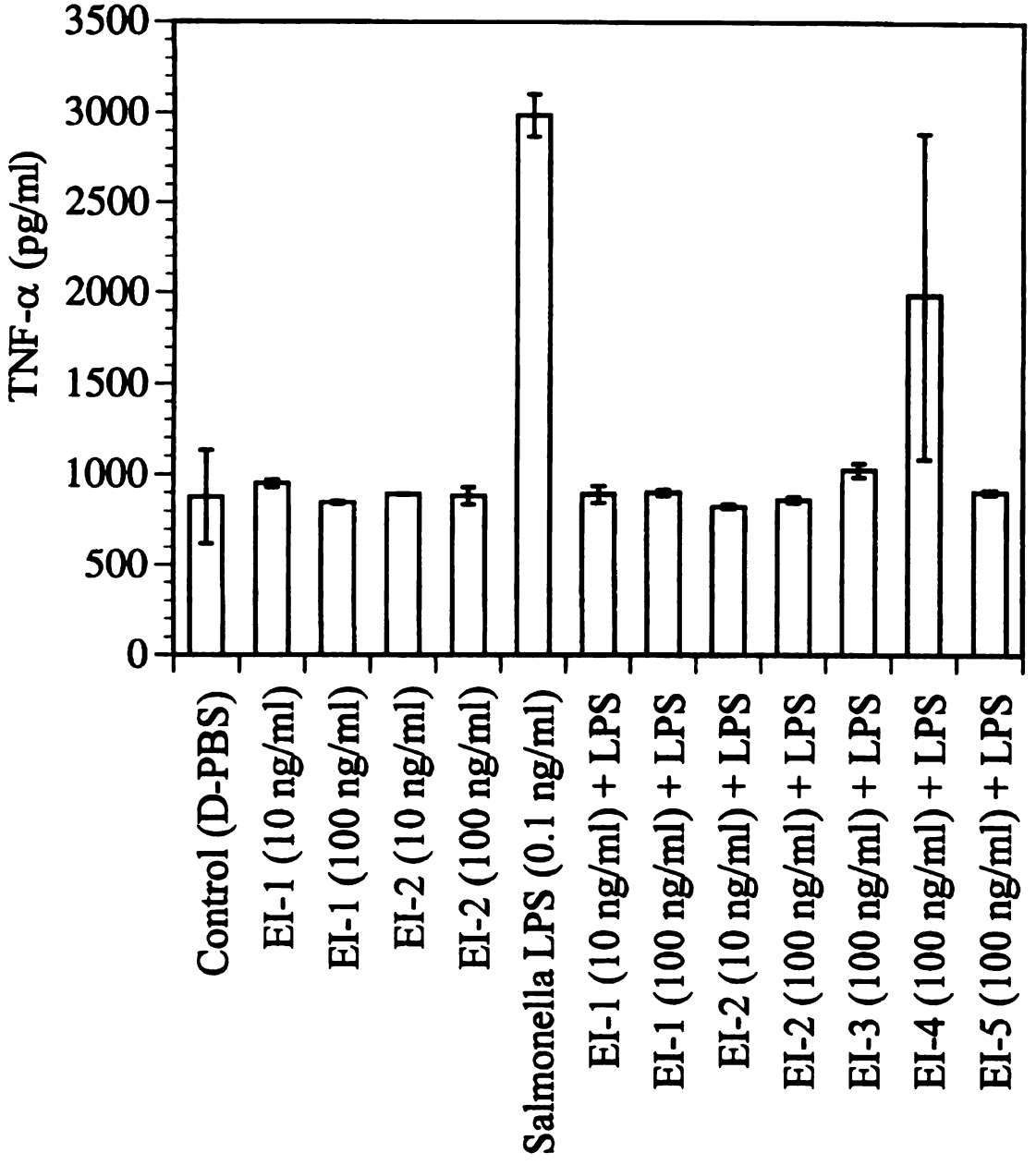


Figure 4.3: Tumor necrosis factor-α (TNF-α) assay. Samples that contain LPS have 0.1 ng/ml *S. typhimurium* LPS.

change upon the addition of EI-1 and EI-2. Furthermore, cell viability (as judged by trypan blue exclusion, Figure 4.4) and cell morphology was not altered after a 10 hour incubation with the synthetic lipids.

One effect of LPS on the properties of membranes that I (Chapter 3) and others (121-124) have observed is their ability to decrease the acyl chain motion (as measured by fluorescence depolarization). If the synthetic lipids were simply acting to increase the acyl chain motions of membrane (i.e. act as membrane 'fluidizers'), then we should be able to observe this by fluorescence depolarization. To test this I used a 1:1 PC:*S. typhimurium* LPS mixture (by weight) and measured the effects of the synthetic lipids on the anisotropy of a fluorescent lipid probe, DPH-PC (Figure 4.5). All five synthetic lipids increased the anisotropy of the membranes (EI-5 > EI-3 > EI-4 > EI-2 > EI-1). This demonstrated that the synthetic lipids have the opposite effect than was expected. They all decrease the acyl chain motion of PC-LPS membranes. My results clearly show that these synthetic lipids do not block the biological activity of LPS by increasing the rate of the large chain reorientation motions of membranes. This indicates that there is not a simple relationship between acyl chain dynamics and biological activity. It is noteworthy, however, that there did appear to be a rough correlation between the number of acyl chains of the synthetic lipids and their ability to change the membrane acyl chain dynamics. For example, the hexa-acyl lipid (EI-5) is the most effective at reducing acyl chain motions while the di-acyl lipids (EI-1 and EI-2) are the least effective.

Proton NMR spectroscopy was used to further examine the effects of the five synthetic lipids on the dynamics and order of PC-*S. typhimurium* LPS mixtures. The resulting NMR spectra are shown in Figures 4.6-4.10. The NMR spectra show that all

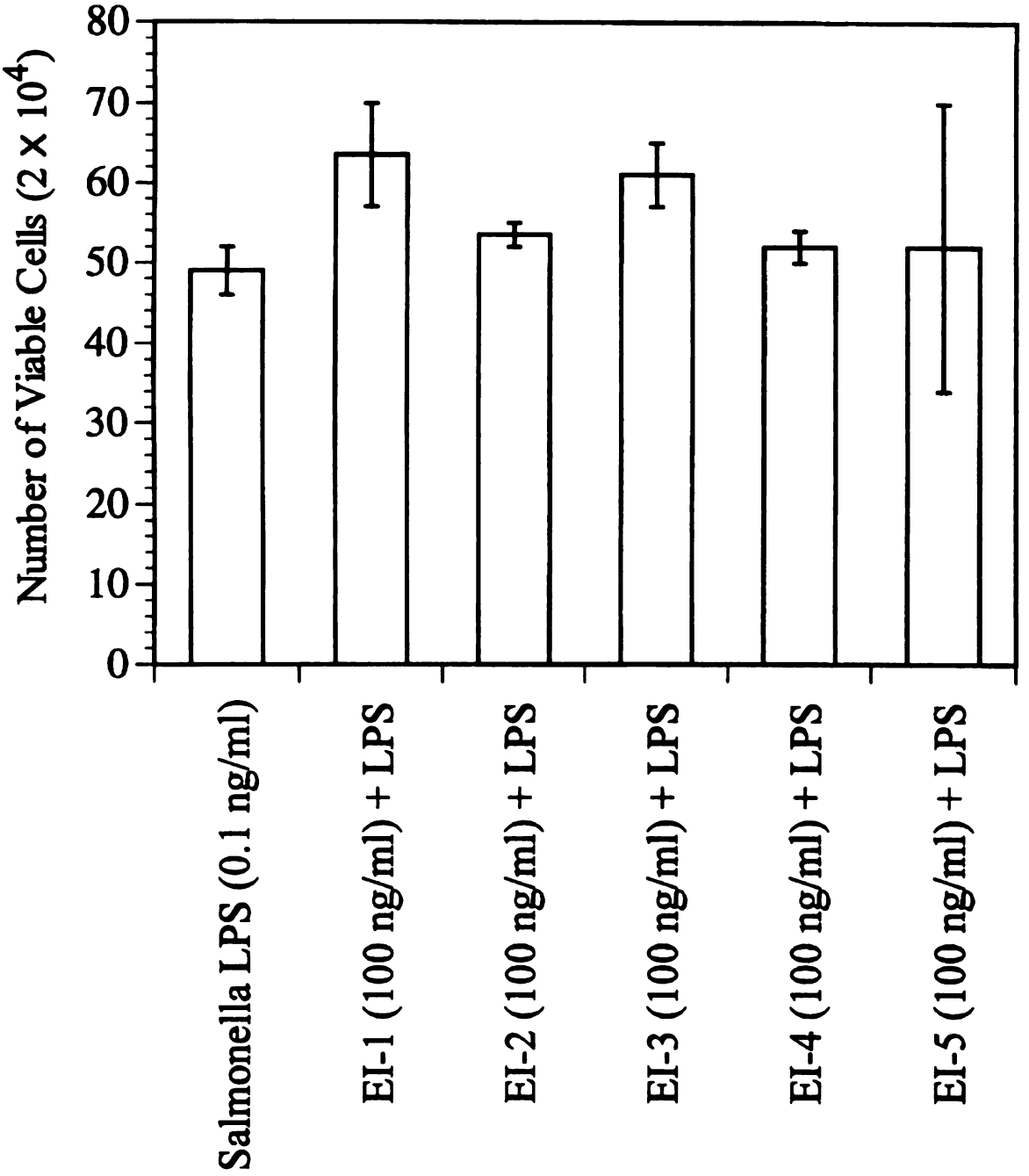


Figure 4.4: Effects of *S. typhimurium* LPS and synthetic lipid-LPS mixtures on RAW264.7 cell viability as judged by trypan blue exclusion. The results are the average of duplicate counts and the error bars show the range of the results.

Figure 4.5: Fluorescence anisotropy of DPH-PC in PC:*S. typhimurium* LPS (1:1 ratio by weight) as a function of synthetic lipid concentration.

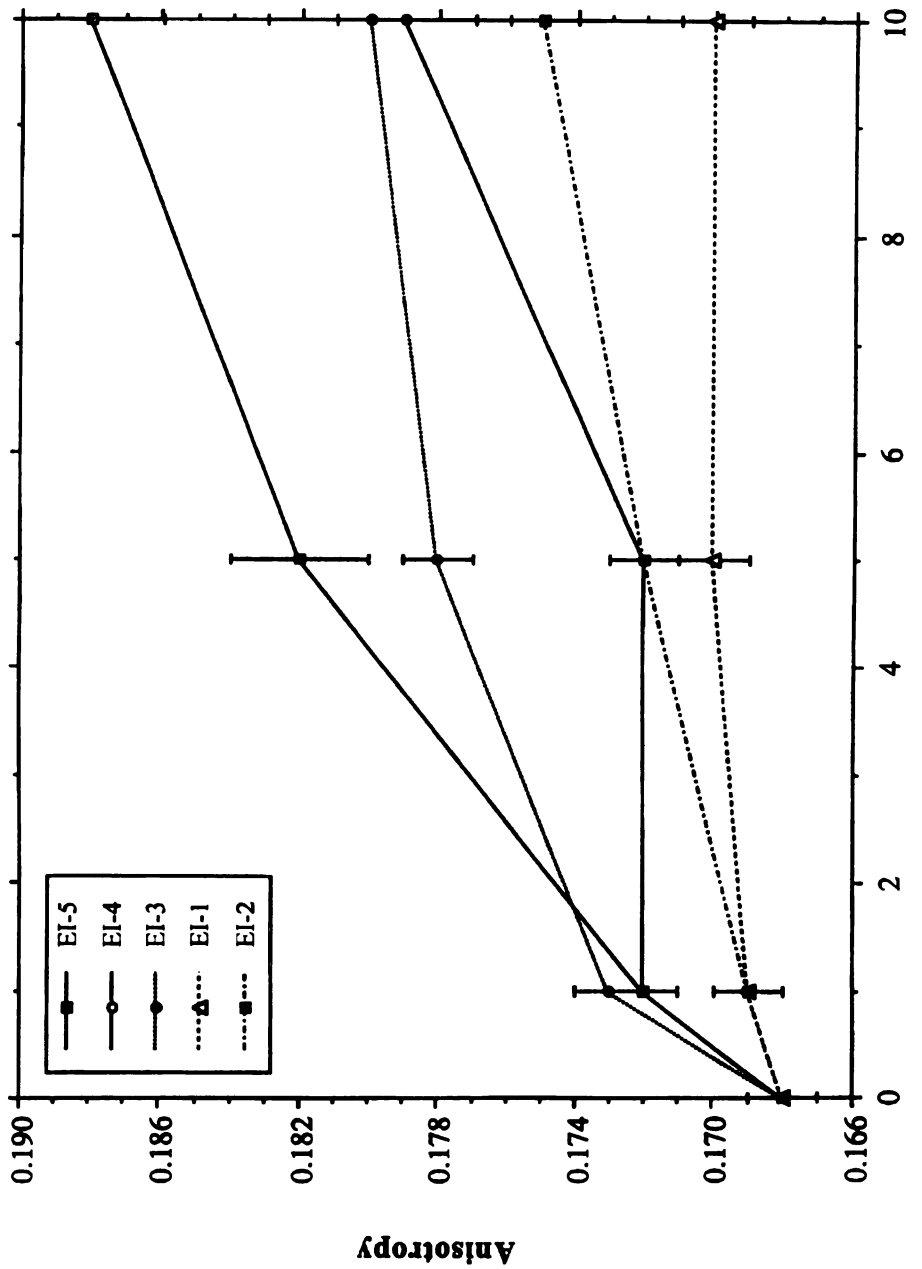

Anisotropy
0.190
0.186
0.182
0.178
0.174
0.170
0.166
0
2
4
6
8
10
Molar Ratio (lipid/LPS)
EI-5
EI-4
EI-3
EI-1
EI-2

Figure 4.5

the synthetic lipids caused an increase in the methyl and methylene proton signals. EI-1, EI-2 and EI-5 resulted in a large increase in the NMR signals while both EI-3 and EI-4 gave only a modest increase. The two methylene proton signals (1.34 and 1.22 ppm) were deconvoluted and the areas of the signals analyzed. The percent change in signal area was calculated and the results are shown in Table 4.1. For the PC-LPS membranes with EI-1, EI-2, and EI-5 added, the percent increase of the two methylene signals is much larger than would be predicted. For example, at a 10:1 EI-1:LPS molar ratio, 0.556 mg of EI-1 was added to a mixture of 1.0 mg PC and 1.0 mg LPS. The addition of EI-1 will give approximately a 42% increase in the number of methylene protons in the sample. However we observe an 81% increase in the total methylene proton signal (102.7% in the 1.34 ppm signal and 42.4% in the 1.22 ppm signal, Table 4.1) in the NMR spectrum (Figure 4.6, D).

To understand the reason for the unexpectedly large increase in the methylene proton signals, we must first understand what the NMR experiment is measuring. In any membrane system there are coexisting regions (domains) of solid-like and fluid-like lipids. Solid-like domains may be especially prevalent in PC-LPS mixtures since we know that LPS increase the order of membranes. However, the proton NMR signals from slowly moving nuclei (such as those from the lipids in solid-like domains) would not be observed due to the extreme signal broadening resulting from dipolar coupling and chemical shift anisotropy. Therefore, we would not expect to see the methylene signals for these rigid lipids. The large increase in the methylene proton signals observed upon the addition of EI-1, EI-2 or EI-5 clearly shows that we are disrupting ('melting') some of those regions and thereby increasing the number of protons observed. These synthetic

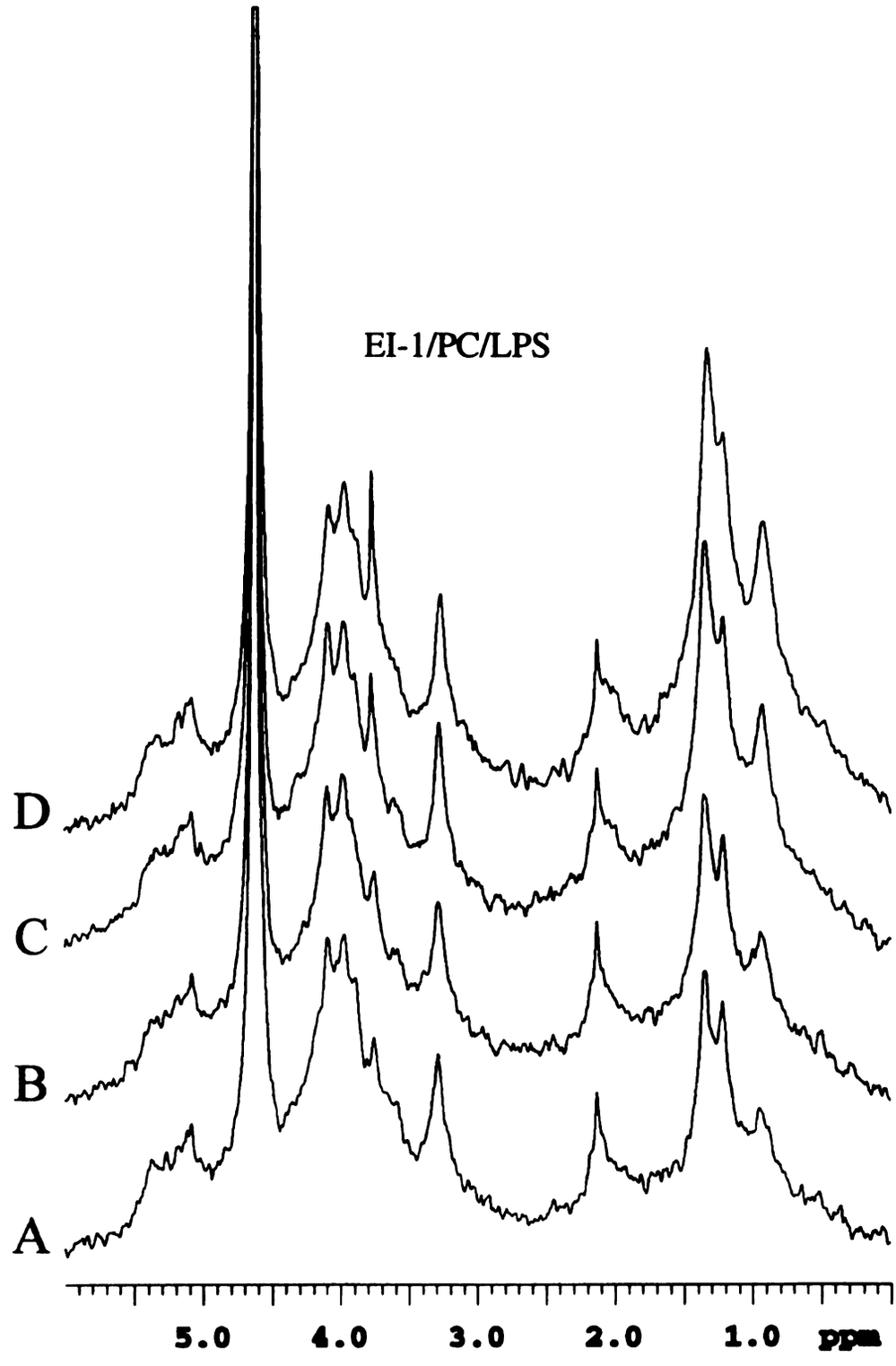


Figure 4.6: ^{1}H-NMR spectra of PC:*Salmonella typhimurium* LPS membranes (1:1 ratio by weight) with a molar ratio of EI-1:LPS of 0 (A), 1:1 (B), 5:1 (C), and 10:1 (D).

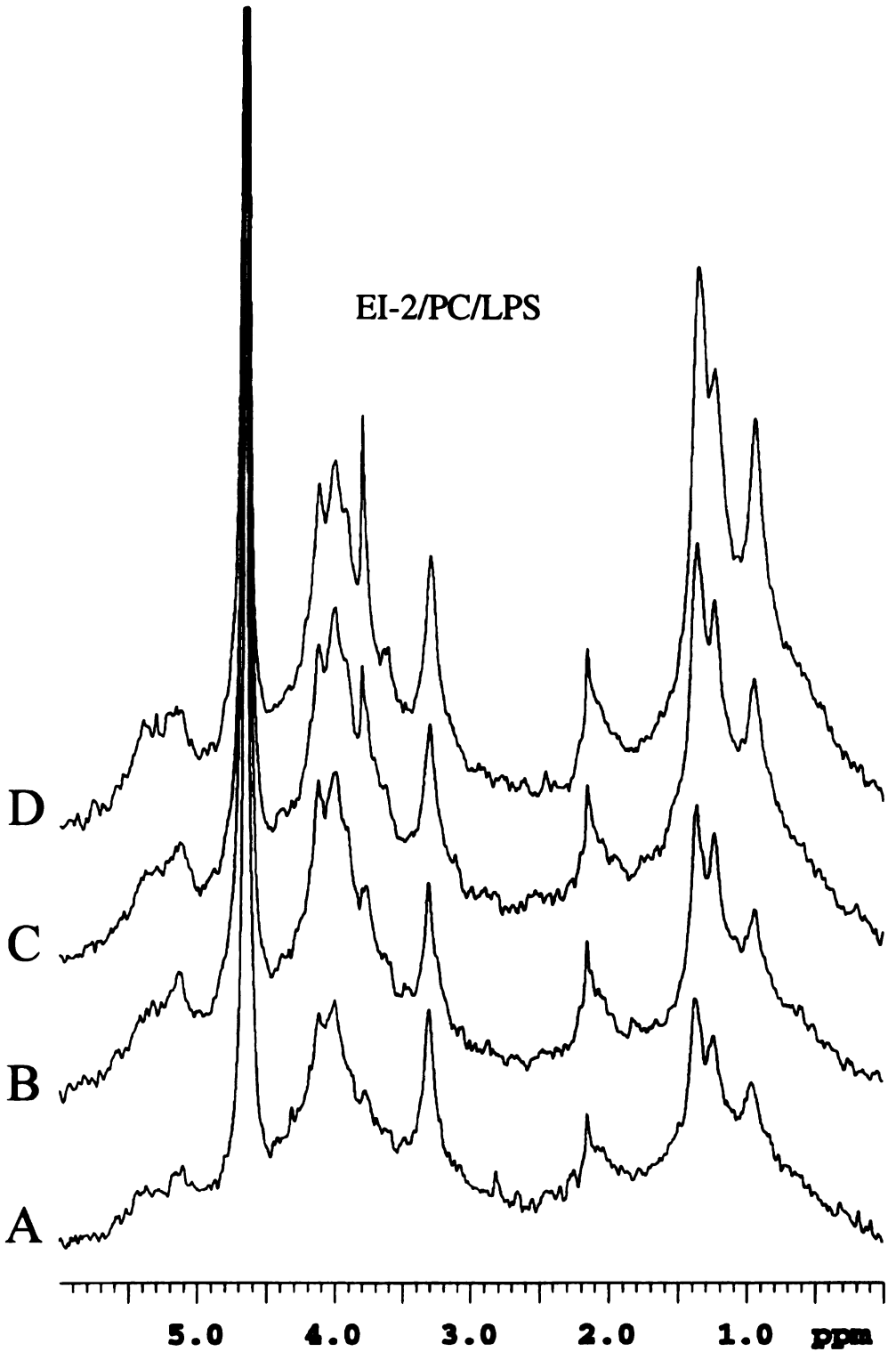


Figure 4.7: ^{1}H-NMR spectra of PC:*Salmonella typhimurium* LPS membranes (1:1 ratio by weight) with a molar ratio of EI-2:LPS of 0 (A), 1:1 (B), 5:1 (C), and 10:1 (D).

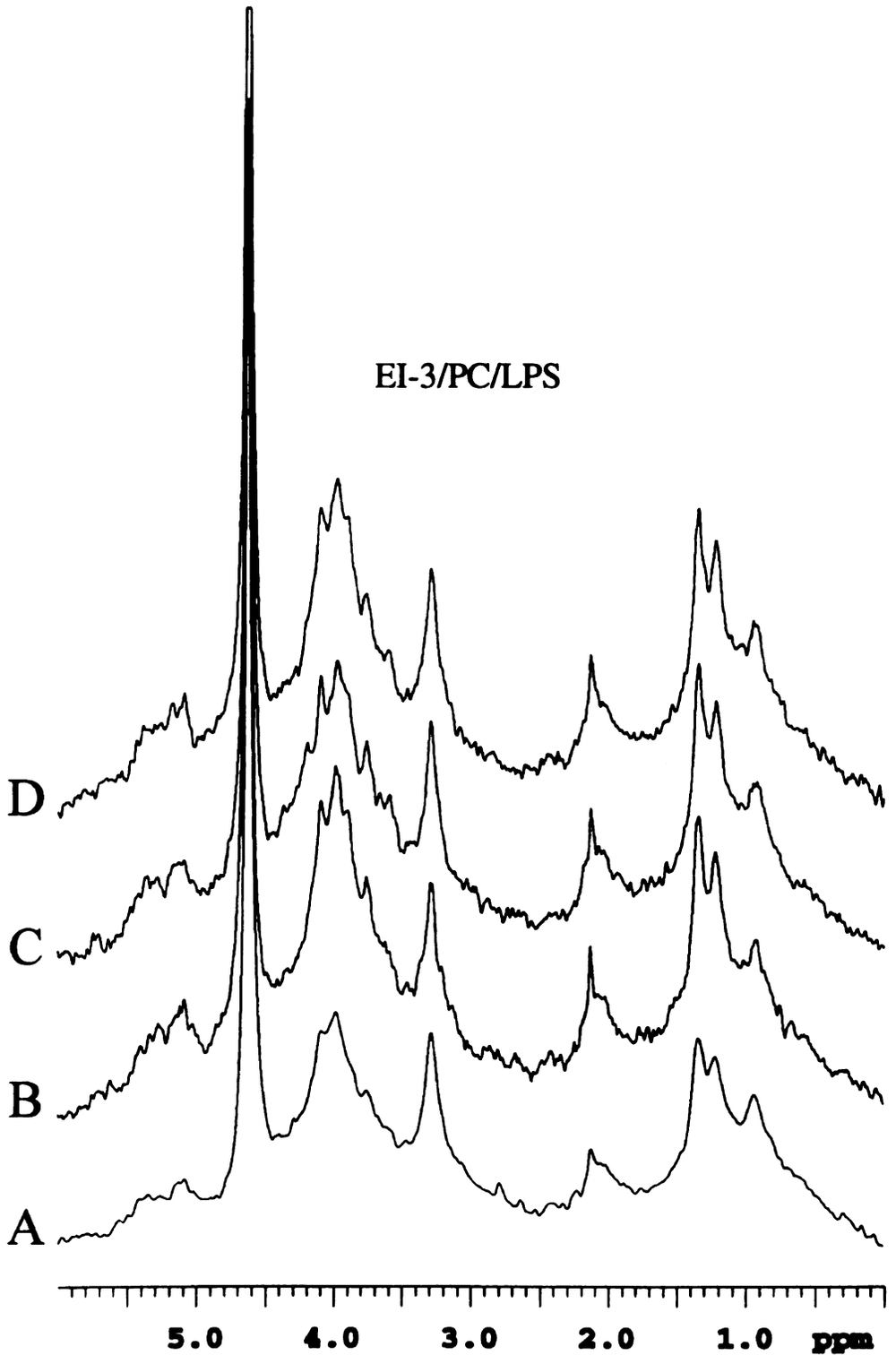


Figure 4.8: ^{1}H-NMR spectra of PC:*Salmonella typhimurium* LPS membranes (1:1 ratio by weight) with a molar ratio of EI-3:LPS of 0 (A), 1:1 (B), 5:1 (C), and 10:1 (D).

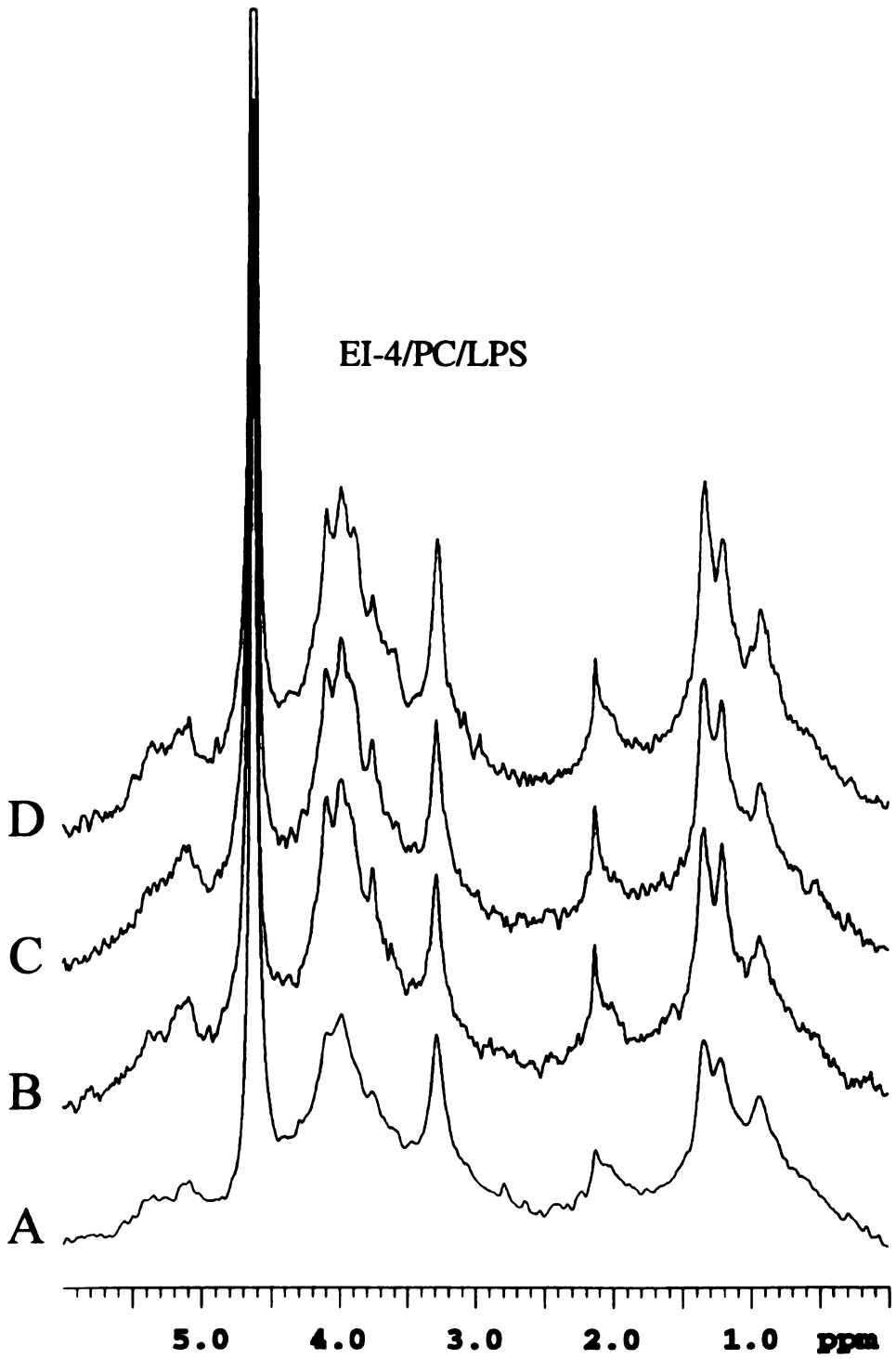


Figure 4.9: ^{1}H-NMR spectra of PC:*Salmonella typhimurium* LPS membranes (1:1 ratio by weight) with a molar ratio of EI-4:LPS of 0 (A), 1:1 (B), 5:1 (C), and 10:1 (D).

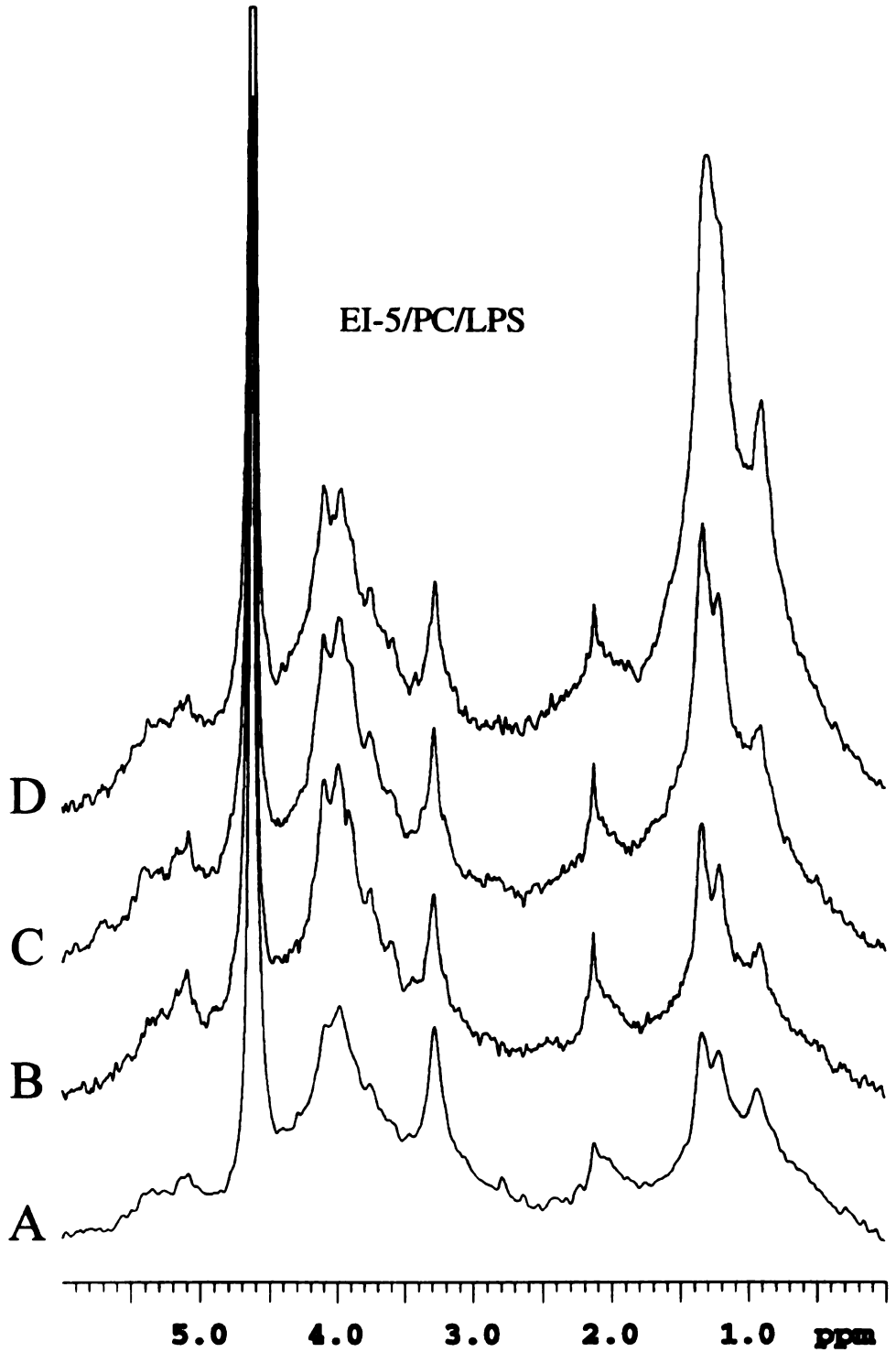


Figure 4.10: ^{1}H-NMR spectra of PC:*Salmonella typhimurium* LPS membranes (1:1 ratio by weight) with a molar ratio of EI-5:LPS of 0 (A), 1:1 (B), 5:1 (C), and 10:1 (D).

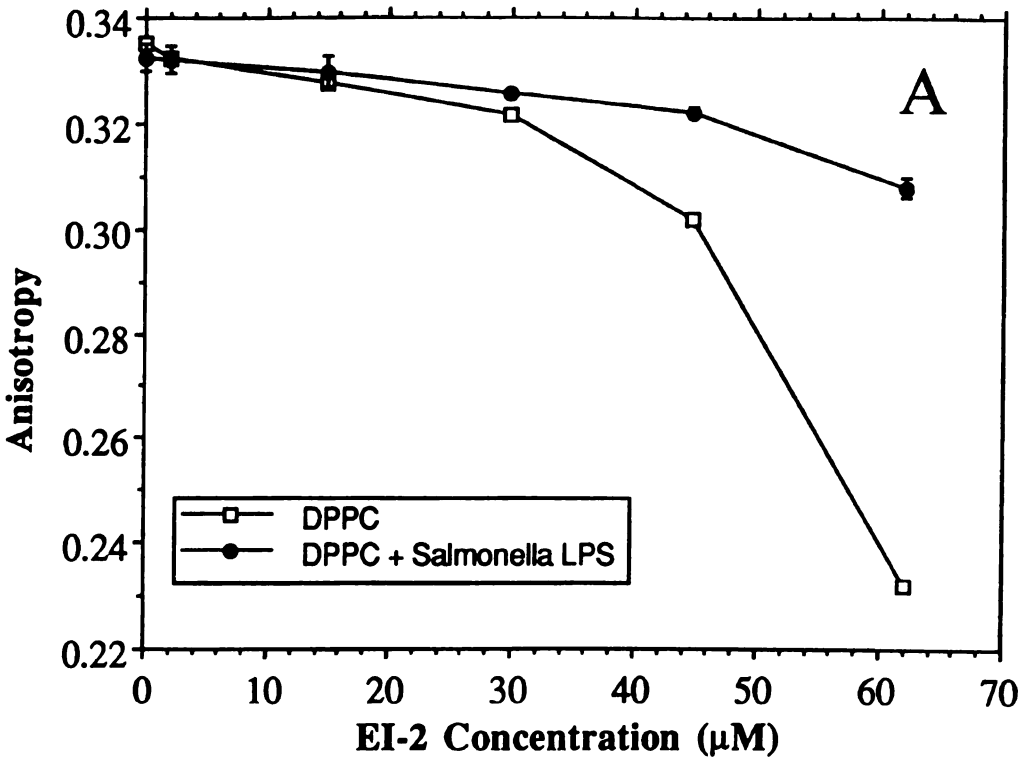
Table 4.1: Percent change in the methylene proton signals upon addition of the synthetic lipids.

Molar Ratio (Lipid:LPS)	Amount of synthetic lipid in sample (mg)	% Change in Area 1.34 ppm signal	% Change in Area 1.22 ppm signal
1:1 EI-1	0.056	21.0	-16.6
5:1 EI-1	0.278	62.6	19.2
10:1 EI-1	0.556	102.7	42.4
1:1 EI-2	0.054	35.6	26.9
5:1 EI-2	0.268	125.8	54.6
10:1 EI-2	0.537	216.4	205.2
1:1 EI-3	0.059	18.1	48.3
5:1 EI-3	0.297	16.6	48.8
10:1 EI-3	0.593	27.5	20.4
1:1 EI-4	0.072	21.3	28.5
5:1 EI-4	0.358	16.2	55.8
10:1 EI-4	0.716	57.0	78.4
1:1 EI-5	0.103	5.8	30.6
5:1 EI-5	0.515	102.6	111.8
10:1 EI-5	1.031	300.9	264.9

lipids are, therefore, causing an increase in the amount of fluid lipids present in the membrane.

The ability of the synthetic lipids to disrupt rigid lipid phases was also observed by fluorescence depolarization measurements of DPPC membranes. Below 42°C, DPPC exists in very highly ordered gel phases. At 42°C, DPPC undergoes a sharp melting transition to the more fluid-like, lamellar liquid crystalline phase (see Figure 1.4). The effects of EI-2 on DPPC and DPPC-*S. typhimurium* LPS mixtures were investigated (Figure 4.11). EI-2 was able to disrupt DPPC and DPPC-LPS as measured by its ability to decrease the fluorescence anisotropy of the membranes at 37°C (Figure 4.11, A). Furthermore, EI-2 broadened the phase transition range and decreased the transition temperature (Figure 4.11, B). This also indicated that EI-2 disrupted the rigid, gel phases

Figure 4.11: Fluorescence anisotropy of tPnA in DPPC and DPPC-*S. typhimurium* LPS (1:1 by weight) membranes with and without EI-2 added. A. Anisotropy of DPPC and DPPC-LPS membranes as a function of EI-2 concentration at 37°C. B. Anisotropy vs. temperature. In B., EI-2 is used at the highest concentration tested in A. Notice the lowering of the transition temperature and the broadening of the phase transition induced by EI-2.


A
Anisotropy
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0
10
20
30
40
50
60
70
EI-2 Concentration (μM)
DPPC
DPPC + Salmonella LPS

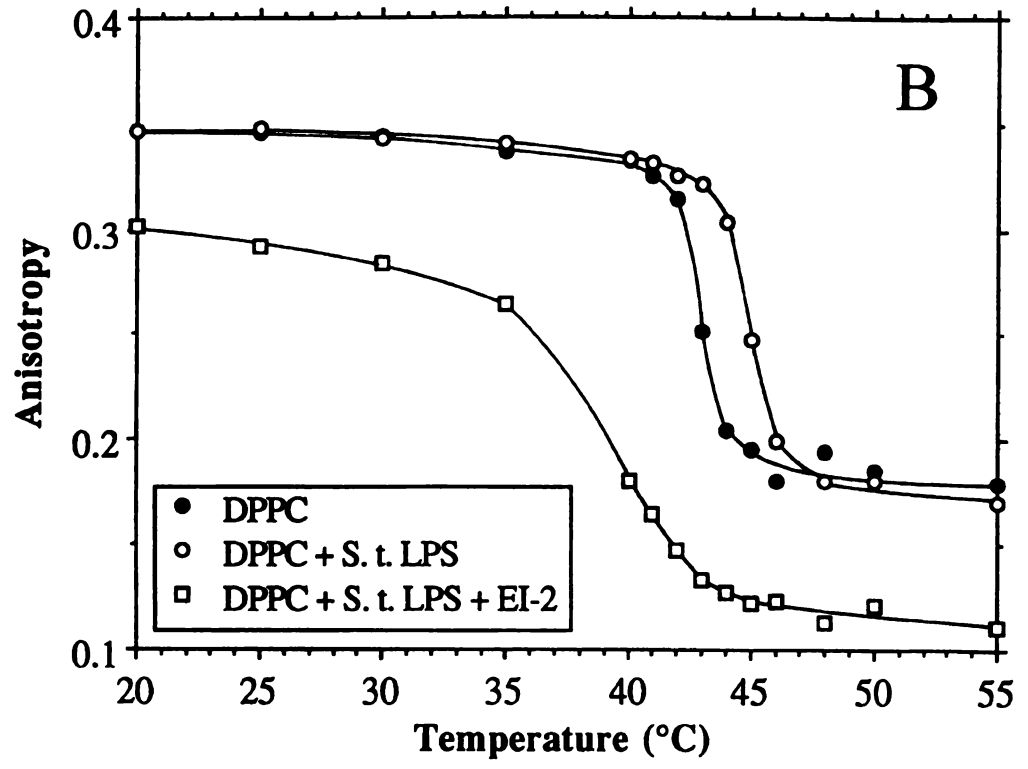

B
Anisotropy
0.4
0.3
0.2
0.1
20
25
30
35
40
45
50
55
Temperature (°C)
DPPC
DPPC + S. t. LPS
DPPC + S. t. LPS + EI-2

Figure 4.11

of DPPC.

The effects of the five synthetic lipids on the *trans-gauche* isomerization rate of the acyl chains of PC-LPS membranes (37°C) were measured by T1 relaxation. As seen previously for PC-LPS membranes (Chapter 3), the magnetization recovery plots for the methylene protons of PC-LPS-synthetic lipid membranes were not single exponential recoveries at all concentrations tested. The data were fit to a double exponential function (Chapter 3, equation 5) and the T1 values obtained are shown in Table 4.2. The synthetic lipids did not give any significant change in the $T1_a$ values for both the 1.34 ppm and 1.22 ppm signals. As in Chapter 3, the $T1_b$ values were not examined quantitatively due to the large error associated with these values. The T1 results demonstrate that the synthetic lipids do not have a large effect on the *trans-gauche* isomerization rate of the acyl chains of the 'fluid' PC-LPS membranes that are observed by NMR spectroscopy.

A previous study by Lill-Elghanian (133) analyzed the effects of the interaction of EI-1 or EI-2 with lipopolysaccharides by determining whether these substances blocked the binding of fluorescently-labeled *S. typhimurium* LPS to RAW264.7 cells. The binding of the LPS (observed by fluorescence microscopy) to the RAW264.7 cells either did not change or increased when EI-1 or EI-2 was added to LPS. This clearly indicated that these substances do not block the binding of the LPS into the cell membranes ruling out the possibility that simple electrostatic blocking was taking place. As discussed above, Jacobs and Price (121) suggested that the binding of LPS to the cell membrane might be mediated by LPS receptors. Many putative LPS receptors have been reported (see Chapter 1). These results clearly show that the endotoxin inhibitors are not competing with these receptors since they do not inhibit binding of LPS to RAW264.7 cells. Yet,

Table 4.2: Effects of the synthetic lipids on the T1 relaxation values for the methylene proton signals in PC-LPS membranes.

1.34 ppm

Molar Ratio (lipid:LPS)	EI-1		EI-2		EI-3		EI-4		EI-5	
	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$
0	0.36	1.0	0.56	6.4	0.56	6.4	0.56	6.4	0.56	6.4
1:1	0.38	1.0	0.46	2.1	0.52	1.6	0.56	6.0	0.56	9.1
5:1	0.50	1.7	0.50	2.8	0.48	2.9	0.48	2.2	0.49	2.0
10:1	0.52	1.6	0.54	4.2	0.55	6.5	0.44	1.4	0.50	1.4

1.22 ppm

Molar Ratio (lipid:LPS)	EI-1		EI-2		EI-3		EI-4		EI-5	
	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$	$T1_a$	$T1_b$
0	0.46	1.4	0.59	6.5	0.59	6.5	0.59	6.5	0.59	6.5
1:1	0.48	1.7	0.45	1.8	0.40	1.0	0.56	-18.8	0.56	-9.3
5:1	0.53	2.0	0.47	1.5	0.56	-4.1	0.52	2.1	0.54	1.5
10:1	0.52	2.0	0.58	-3.5	0.54	3.9	0.54	17.6	0.52	1.7

they are able to block the biological activity of LPS as measured by the LAL and TNF induction assays. This indicates that the mode of action of the inhibitors is not by blocking the interaction of LPS with surface of the cells.

Together, these results are consistent with the endotoxin inhibitors functioning to alter the ability of LPS to induce membrane perturbations. This could occur by either (1) direct disruption of the LPS aggregate structure, thus altering the ability of LPS to induce a membrane perturbation, or (2) change of the membrane properties that offset the perturbations induced by LPS. In the former scenario, we would expect that a relatively small quantity of the synthetic lipids is needed to block activity. Intercalation of an approximately equal molar ratio of the synthetic lipid into the LPS aggregate should alter its aggregate structure and thus block activity. However, we observe that the biological activity is blocked at a much higher molar ratio of the synthetic lipids. For example, at

10 ng/ml EI-1 and 0.1 ng/ml LPS, the molar ratio is approximately 1800:1 EI-1:LPS (assuming an average molecular weight of 15000 g/mole for LPS). The high level of the synthetic lipids required indicates that the second scenario is the predominant mechanism by which they are inhibiting the biological activity of LPS.

Conclusion

I definitively show that the five synthetic lipids (EI-1 through EI-5) block the ability of LPS to activate the clotting enzyme cascade of the *Limulus* amebocyte lysate and to induce the secretion of TNF from macrophages. My results also demonstrate that the synthetic lipids do not just simply cause an alteration in the acyl chain mobility of membranes, they also induce a disruption of the domain structure (order) of the membrane. I propose that the perturbation in the membrane dynamics and order elicited by the synthetic lipids is the mechanism by which they inhibit the biological activity of LPS. Other possible mechanisms of inhibition by the synthetic lipids have also been explored. The synthetic lipids do not show any signs of toxicity for the RAW264.7 cells at the concentrations used thus eliminating the possibility that these lipids simply block the TNF-α response by killing the macrophage cells. The synthetic lipids could also inhibit the biological activity of LPS by blocking the electrostatic interaction of LPS with the macrophages. This possibility was explored for EI-1 and EI-2 by measuring the binding of fluorescent LPS to the cells (133). EI-1 and EI-2 did not decrease the amount of LPS bound to the macrophages, thus demonstrating that the inhibitors are not blocking the binding of LPS to the macrophages. These results clearly show that these possibilities are not responsible for the inhibition of the biological activity by the

synthetic lipids. This is consistent with the hypothesis that EI-1 – EI-5 function by affecting the properties of the host cell membrane.

My results indicate that the change in the lipid domain structure induced by the synthetic lipids is a major contributing component in the inhibition of a physiological response by LPS. Furthermore, the five synthetic lipids reduce the biological activity of LPS by altering the domain structure of the membranes, not simply by increasing the lipid acyl chain motion as previously suggested (133). Furthermore, the large concentration of the synthetic lipids required to suppress the biological activity of LPS suggests that they function by offsetting the membrane perturbations induced by LPS.

From our results, it should be expected that many other unrelated molecules may be able to suppress the biological activity of LPS if they are able to interact with the cell in such a way as to counteract the effects of LPS on the properties of the membrane. In fact, suppression of the physiological effects of LPS is observed for such diverse lipophilic structures as *Rhodobacter sphaeroides* lipid A (which has a very unusual fatty acid structure) (97-99), lipid X and lipid X derivatives (180-182), polymyxin B (183, 184), tetrahydrocannabinol (THC, the active ingredient of marijuana) (185), ethanol (186, 187), and dimethyl sulfoxide (188). Clearly, the link between the effects LPS has on the dynamics and order of membranes and its ability to elicit a biological response is very complicated and will not be explained by a simple model.

Chapter 5

Summary and Perspective

Since Pfeiffer first discovered lipopolysaccharides over 100 years ago, an enormous amount of research has been devoted to understanding its properties. Perhaps Berry (189) summarized it best when he wrote: "An important reason why endotoxin has been the object of so much research is its diversity of action. It poses challenges to chemists, physiologists, immunologists, microbiologists, pharmacologists, endocrinologists, biochemists, anatomists or structural biologists, pathologists, internists, surgeons, infectious disease specialists and others. A substance with an appeal as broad as this is rare in science." The interest in LPS continues unabated. Even as this dissertation is being written, approximately 200 journal articles that study, use, or are related to LPS research are being published every month.

Despite this intense multidisciplinary scrutiny, the molecular mechanism underlying the LPS-induced activation of the immune systems of animals remains elusive. The severe immune response elicited by LPS is, in large part, responsible for the physiological effects characteristic of Gram-negative septicemia. Gram-negative septicemia continues to be a major clinical problem and results in the death of thousands of individuals each year. Understanding how LPS elicits such a severe immune response is critical to developing effective therapies for this problem. The ever increasing number of antibiotic resistant bacterial strains only adds urgency to this issue. Moreover, understanding LPS's potent immuno-stimulatory power may lead to therapies for immune compromised individuals such as those undergoing chemotherapy.

I think that it is fair to say that anyone who has examined all the aspects of LPS would agree that its mechanism of action is tremendously complex. Accordingly, the LPS literature is inundated with seemingly unrelated or even contradictory results. To

date, the two step model proposed by Jacobs and Price (121) has been the only model describing the molecular mechanism of the LPS-host cell interaction that is consistent with the bulk of the known data on LPS (see Figure 1.11). This model divides the LPS-host cell interaction into two distinct steps. The first step is the electrostatic interaction of LPS with the surface of the immune cell membrane. The second step is the rearrangement of the LPS so that the lipid A region inserts into the membrane bilayer. They propose that there may be proteins (receptors) that facilitate the binding and insertion of LPS into membranes. Furthermore, they propose that the activation of immune cells by LPS is a consequence of the insertion of LPS into the membrane and not the binding to the surface of the membrane.

Lets examine the aspects of this model in light of the data presented in this dissertation.

Electrostatic Interaction of LPS with Membranes. For more than a decade, the concept of an LPS-specific receptor has dominated the LPS literature (see Chapter 1). In general, it is thought that there is a membrane associated protein that, when bound to LPS, induces a signal transduction cascade to activate the immune cell. A true LPS receptor would need to have the properties of being specific for lipid A (i.e. can accept only limited structural diversity within lipid A) and, most importantly, be able to transmit a functional signal across the membrane in response to binding of LPS.

The initial interaction of LPS with cell membranes is electrostatic in nature as shown by the experiments of Carr and Morrison (118, 119) and Jacobs and co-workers (120). Furthermore, there is little doubt that proteins may be involved in this interaction. In fact, this is quite reasonable since proteins are such a major component of membranes.

However, the question that must be addressed is whether or not these interactions are specific and result in a signal being transmitted across the membrane. Our results from Chapter 2 demonstrate that the interaction of LPS with immune cells does not require a highly conserved lipid A structure. We found that lipid A from species of *Rhizobium* had several of the biological activities typical of enteric lipid A despite their highly unusual structure. They not only have unique headgroups, which would be involved in the electrostatic interaction with the membrane, but they also have very unusual fatty acid structures. The ability of these LPS to elicit a biological response is especially amazing when we consider the fact that the lipid A from *Rhodobacter sphaeroides*, which only differs in its fatty acid structure (Figure 1.10), is completely devoid of biological activity. This clearly demonstrates that a specific interaction of lipid A with the host cell membrane is not a requirement for biological activity. In fact, the most promising of the LPS 'receptors' found to date, CD14, has been shown to be very non-specific for LPS. It binds to a wide range of polyanionic molecules (145, 146). Furthermore, CD14 is not a transmembrane protein and therefore, can not transduce a signal across the membrane in response to LPS. Therefore CD14 can not, in the true sense of the word, be called an LPS receptor.

Unfortunately, the term receptor has been used very loosely in the LPS literature. Of course, all molecules interact with each other to some extent. In the case of LPS, its charge and amphiphilic nature make some of these interactions favorable. Those proteins that have exhibited a favorable interaction with LPS are termed receptors even though they may not be truly specific or functional receptors. In this loose definition of receptors, almost any molecule, whether a protein or not, could potentially be called an

LPS receptor. The numerous LPS receptors identified so far may actually bind to LPS as a result of serendipitous favorable molecular interactions and not because they were designed to seek out LPS and transmit a signal across the membrane in response to it.

So what is the role of these LPS 'receptors'? Jacobs and Price suggested that some proteins on the surface of the membrane might act to facilitate the incorporation of LPS into the membrane (121). This appears to be a likely scenario. In fact, lipopolysaccharide binding protein and CD14 have been shown to facilitate the incorporation of LPS into phospholipid bilayers (147-149). The incorporation of LPS into membranes by these proteins may simply be a result of their ability to efficiently bring LPS into close proximity to the membrane.

From the discussions above, we can see that the binding of LPS to the cell surface is not the step that induces a cellular signal. Instead, the events that occur when LPS is incorporated into the membrane are the essential points that must be addressed in order to understand how LPS activates immune cells.

Effects of LPS on Membranes. In the two step model, Jacobs and Price proposed that the activation of immune cells by LPS is a consequence of the insertion of LPS into the membrane (121). They, as well as others, demonstrated that LPS is capable of decreasing the 'fluidity' of membranes (121, 123, 124, 160, 161). However, they fall just short of explaining how the insertion of LPS into the cell membrane results in the activation of the cell.

Cellular membranes are extremely complex. They exist as an aggregate with differing types and degrees of motions and order. The dynamics and order of membranes have a tremendous influence on the function of the membrane (see Chapter 1). They can

control the activity of membrane-associated proteins, the flux of enzyme substrates and protein-protein interactions. This, in turn, will affect the function of the cell. However, the dynamics and order of membranes are not constant. Instead, they are sensitive to environmental conditions such as temperature and pH. Furthermore, molecules that interact with membranes will also affect its properties. Thus, these molecules will also have the potential to alter the normal functions of the membrane and the cell.

The ability of LPS to incorporate into cellular membranes may result in a change in the function of the membrane. If this was involved in the mechanism of LPS action, then we should be able to observe changes in membrane properties induced by LPS. In Chapter 3, we described the changes in membrane properties induced by LPS. We found that LPS induced dramatic changes in both the dynamics and the order of phospholipids. Furthermore, the results obtained from synthetic lipid probes (Chapter 4) indicate that membrane perturbations are central to the physiological responses elicited by LPS.

My data also indicate that the relationship between the effects of LPS on membrane properties and its ability to elicit a biological response is not simple. We show that there is not a linear relationship between the biological activity of LPS and the changes in membrane acyl chain motion. In hindsight, its seems unreasonable to expect a simple relationship to exist. Membrane dynamics and order are extremely complex and interrelated. There is a complex interplay between membrane dynamics and order and by changing one parameter, all of the properties are affected. Moreover, our understanding of the relationship between these changes in membrane properties and the function of the membrane are still in its infancy. We can not yet fully predict the functional changes that may be expected from perturbations in membrane properties.

Model for the Mechanism of LPS-Induced Immune Cell Activation. The two step model proposed by Jacobs and Price remains the only molecular model to describe the LPS-host cell interaction. My results are consistent with this model and also extends some of the original points of this model:

1. The first step is the electrostatic interaction of LPS (or lipid A) with the cell membrane. This interaction is not highly specific and may involve a variety of membrane molecules, including membrane proteins. This non-specific interaction does not result in a cellular signal.
2. LPS intercalates into the cell membrane.
3. The insertion of LPS results in the perturbation of the membrane dynamics and order. The extent and nature of the membrane perturbations is dependent on the physicochemical properties of the individual LPS molecules and of its aggregates.
4. The membrane perturbations induced by LPS results in a functional change in the membrane and this, in turn, triggers the cellular response such as the secretion of cytokines.

This last point requires further discussion. To the best of my knowledge, this is the first time that membrane perturbations have been proposed as *the* decisive molecular event that leads to the activation of immune cells by LPS. How might this occur? The data presented in this dissertation clearly demonstrated that LPS affect both the dynamic and organizational state of membranes. In Chapter 1, we discussed the physiological significance of membrane dynamics and order. The activities of many membrane-associated proteins are dependent upon the physical state of the membrane. Furthermore,

the degree of connectivity of domains can create functional areas in the membrane and can control the flux of substrates and protein-protein interactions. Two particularly well studied membrane proteins (with respect to membrane effects) are phospholipase A_2 (PLA_2) and protein kinase C (PKC). Both of these proteins are important components of signal transduction pathways. PLA_2 activity is affected by membrane 'fluidity' (45), bilayer packing stress and defects (50, 51), and the formation of solid-like lipid domains (59). Interestingly, LPS alters all of these membrane properties. I found that LPS decreases lipid acyl chain motion ('fluidity'), induces regions of fast motion (membrane defects), and alters the domain organization of membranes. As would be predicted from these effects, LPS is known to activate PLA_2 (190). PKC activity is also dependent upon domain formation (60) and has been shown to be closely associated with the hexagonal phase propensity of the membrane (52). With respect to this last point, Brandenburg and co-workers have found that the biological activity of LPS is correlated to its ability to form inverted phases such as hexagonal phases (7, 129). Toxic LPS favored inverted phases. As might be expected, LPS also activates PKC (191, 192). While the examples of PLA_2 and PKC illustrate how the perturbations induced by LPS can alter the membrane function, they only begin to show the potential for these types of effects. As our knowledge of the physiological significance of membrane dynamics and order increases, so will our knowledge of how LPS induced membrane perturbations may influence cellular functions.

The extended two step model described above has some important predictions. First, this model does not necessitate the direct interaction of LPS with membrane proteins in order to elicit a biological response. Second, any lipophilic molecule may

affect the biological activity of LPS if this molecule is capable of offsetting or enhancing the membrane perturbations induced by LPS. Finally, the ability of LPS to elicit an immune cell response may also be altered by environmental parameters that affect membrane dynamics and order, such as temperature, pressure, ionic strength, and pH. It should also be recognized that lipophilic molecules or environmental parameters might be able to mimic some of the biological responses induced by LPS.

We already see evidence supporting these predictions. Many lipophilic molecules are capable of reducing the biological activity of LPS. These include *R. sphaeroides* lipid A (97-99), lipid X and lipid X derivatives (180-182), polymyxin B (183, 184), tetrahydrocannabinol (THC) (185), ethanol (186, 187), and dimethyl sulfoxide (188). While we do not know the exact mechanism by which they decrease the biological activity of LPS, their ability to interact with the membrane is consistent with our model. Temperature has also been shown to affect tumor necrosis factor (TNF) secretion from macrophages. Increasing the temperature of macrophages, which would increase the motion of the membrane, results in a decreased production of TNF-α in response to LPS (174-176). The opposite effect is observed when the temperature is lowered. This treatment would decrease the motion of the macrophage membrane (analogous to the addition of LPS to membranes). Lowering the temperature increases the production of TNF-α from LPS-stimulated macrophages (177, 178).

Other typical biological activities induced by LPS can be linked to the involvement of membranes. These include the *Limulus* amebocyte lysate (LAL) assay, thrombosis, and B-lymphocyte activation. The LAL assay utilizes the blood clotting cascade of the horseshoe crab as a sensitive and specific measure of LPS toxicity. The

blood clotting cascade of the horseshoe crab is highly homologous to the mammalian blood clotting mechanism and they are believed to be evolutionarily related (193). In mammals, the enzyme complexes responsible for clotting depend on and assemble in membranes (194). Calcium ions are essential for the proper folding of the membrane binding domains of these proteins and are also involved in the cross-linking of the acidic protein residues to the phosphates on the membrane lipids (195). Clearly, the membrane is a critical component of the clotting cascade in mammals. Unfortunately, the role of the membrane in the horseshoe crab clotting cascade has been largely ignored. There is, however, strong evidence that at least some of the enzymes of this cascade are also dependent on membrane interactions. Calcium ions are needed for the clotting cascade analogous to the mammalian clotting cascade (196). Lui et al. have also shown that the purified proclotting enzyme of this cascade requires detergent to be active (197), indicating that this enzyme also requires an interaction with a membrane for activity. We believe that the physical properties of the membranes with which the enzymes interact will affect the activity of the enzymes of the clotting cascade. The ability of LPS to alter the dynamics of these membranes will thus result in an alteration of the clotting cascade.

LPS also affects B-lymphocytes. LPS acts as a mitogenic signal for the induction of B-lymphocyte proliferation and differentiation into antibody secreting plasma cells. When LPS binds to B-lymphocytes, it induces a major lateral rearrangement of the plasma membrane (capping) (121, 198). The LPS response of B-lymphocytes has garnered a lot of attention since the discovery of a genetically altered mouse strain (C3H/HeJ) whose B-lymphocytes were hyporesponsive to LPS (199). It was originally believed that the reason for the low response to LPS was due to a missing or altered LPS

receptor. However, binding of LPS to C3H/HeJ B-lymphocytes was not altered and no missing receptor has been identified (199, 200). One difference between the normal- and hyporesponsive B-lymphocytes is their membrane composition. Chaby et al. (201) noted a change in the ganglioside composition in C3H/HeJ B-lymphocytes. A later study by Yohe, et al. (202), demonstrated that there is a change in the structure of the C3H/HeJ B-lymphocyte membrane that alters the surface accessibility of the gangliosides. These studies clearly show that LPS hyporesponsive B-lymphocytes have altered membrane compositions. The decreased response of these cells is consistent with the idea that the membrane structural changes alter the membrane perturbations induced by LPS and, thus, affect the cell's ability to respond to LPS.

The findings of this dissertation highlight the importance of understanding LPS-membrane interactions. My research has advanced our understanding of the effects LPS has on the properties of membranes. However, we are only just beginning to fully understand LPS-membrane interactions. This area of LPS research needs more attention in the future. Some experiments should focus on understanding the effects of other LPS structures (such as *Rhizobium* or *Rhodobacter* LPS) on the properties of membranes. In addition to the experiments described in this dissertation, I think that deuterium NMR may be very powerful for this purpose. With this technique, specific lipid species can be ^{2}H-labeled, thus allowing a definitive measure of the properties of individual lipid species in a complex mixture. Furthermore, it may be possible to isolate ^{2}H-labeled lipid A from bacteria by using minimal media with a deuterated carbon source. Magic angle spinning NMR spectroscopy should also be explored since it is able to resolve many of the methylene signals in the lipid acyl chains (203). This would allow an even more detailed

look at the motions of the lipid acyl chains. My results showed that LPS induces domain formations in membranes. This also needs to be investigated further. Fluorescence microscopy on model membranes may provide useful information related to LPS induced domains. This could be done with multilamellar, bilayer (black lipid membranes), monolayer, or cellular (such as erythrocyte) membranes (see ref. 14-21, 27, 36, 39, 40). Fluorescence recovery after photobleaching (FRAP) has also provided information on lipid domains and could be used (see ref. 36, 37, 61). Furthermore, FRAP would measure the change in the lateral diffusion of lipids in response to LPS. This would also be a good parameter to assess the changes induced by LPS. Finally, I think that the effect of LPS on different lipid species needs to be analyzed. One previous study addressed this issue (160). Given the negative charge of lipid A and its high degree of order, we might expect that it would have a strong preference to associate with negatively charged phospholipids (due to ionic bridging) and saturated lipids (due to more favorable van der Waals interactions with the acyl chains). The preference of lipid A to associate with specific lipid species will surely be an important component of its ability to elicit membrane perturbations.

These experiments will allow future investigators to further our understanding of LPS-membrane interactions. In turn, this will help us to understand how LPS elicits such diverse and severe biological responses. In this quest, we must continually remember that LPS is just a lipid and the key to understanding its effects lies in its ability to interact with other lipids. After all, the main function of LPS is to serve as a barrier on the surface of Gram-negative bacteria to protect the cell from environmental stress. It is unlikely that LPS was designed to wreck havoc on the immune systems of animals. One

thing that is certain, however, is that the interaction of LPS with animals is extremely complex and will capture the attention of many more researchers in the years to come.

APPENDIX

APPENDIX

Synthesis and Characterization of Five Synthetic Lipids

Introduction

In Chapter 4, I describe the results obtained from five synthetic lipids (EI-1 to EI-5) (see Figure 4.1). These lipids were found to be good inhibitors of the biological activities of LPS. My results indicate that these lipids function by altering the physical properties of membranes and offset the perturbations induced by LPS.

To understand how these lipids affect membrane properties, one must first understand their physical properties. The synthesis of EI-1 and EI-2 was described previously (174). In this report, I describe the synthesis, purification and characterization of EI-3, EI-4 and EI-5. I also characterize the physical properties of all five of the lipids by differential scanning calorimetry (DSC), x-ray powder diffraction, and proton NMR T1 relaxation measurements.

Materials and Methods

Mass Spectrometry. Electrospray ionization mass spectrometry was performed on a Fisons Platform instrument using a 4:1 $CHCl_3$:methanol mobile phase. Samples were prepared in the same mobile phase with 1% formic acid added and positive ion spectra were recorded. Spectra were processed by subtracting the baseline and smoothing the data.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H-NMR spectra were recorded in $CDCl_3$ on a Varian 500 MHz VXR spectrometer. EI-3 and EI-4 required the addition of a couple of drops of deuterated methanol to dissolve the samples. The $CDCl_3$ solvent peak (7.24 ppm) was used as the reference. Spin-lattice (T1) relaxation measurements were performed on a Varian 300 MHz spectrometer using the inversion recovery

technique (169). An 8 s delay was used to allow for complete equilibration of the nuclei. The temperature was allowed to equilibrate for at least 15 min before the measurements were taken. All of the samples for T1 measurements were dissolved in the same stock solution of 1:1 $CDCl_3$:CD_3OD. This solution was used because it was the most polar solvent system that would dissolve all of the lipids.

Differential Scanning Calorimetry (DSC). DSC measurements were made on a Microcal-2 high sensitivity scanning calorimeter at 60°C/hr. Approximately 5 mg of synthetic lipids were hydrated in water (1.5 ml) and multiple scans (up to 5) were recorded from 5°C to 98°C.

X-Ray Diffraction. X-ray diffraction patterns were recorded on a Rigaku rotaflex rotating anode operating at 45 kV and 100 mA. The raw x-ray beam was collimated with 1/6° slits and the copper K_α line (1.54 Å) was selected by a monochromator. Dry samples were deposited on a microscope slide in a thin film and the x-ray diffraction pattern obtained in the reflection mode. Samples in solution were placed into 0.7 mm glass capillaries (Charles Supper Co.) and mounted with a home-built capillary holder. Diffraction patterns were obtained in the transmittance mode. All diffraction patterns were obtained at room temperature. A one-dimensional scanning detector was used to measure the reflected radiation.

Synthesis and Purification of EI-3, EI-4 and EI-5

EI-3 was synthesized by the scheme shown in Figure A.1. Tris(2-aminoethyl)amine and approximately 4 equivalents of fatty acid methyl esters (palmetic acid, technical, methanolized in 5% HCl in methanol) were mixed in ethanol and refluxed for 3 hours. The solvent was evaporated under reduced pressure and the remaining white

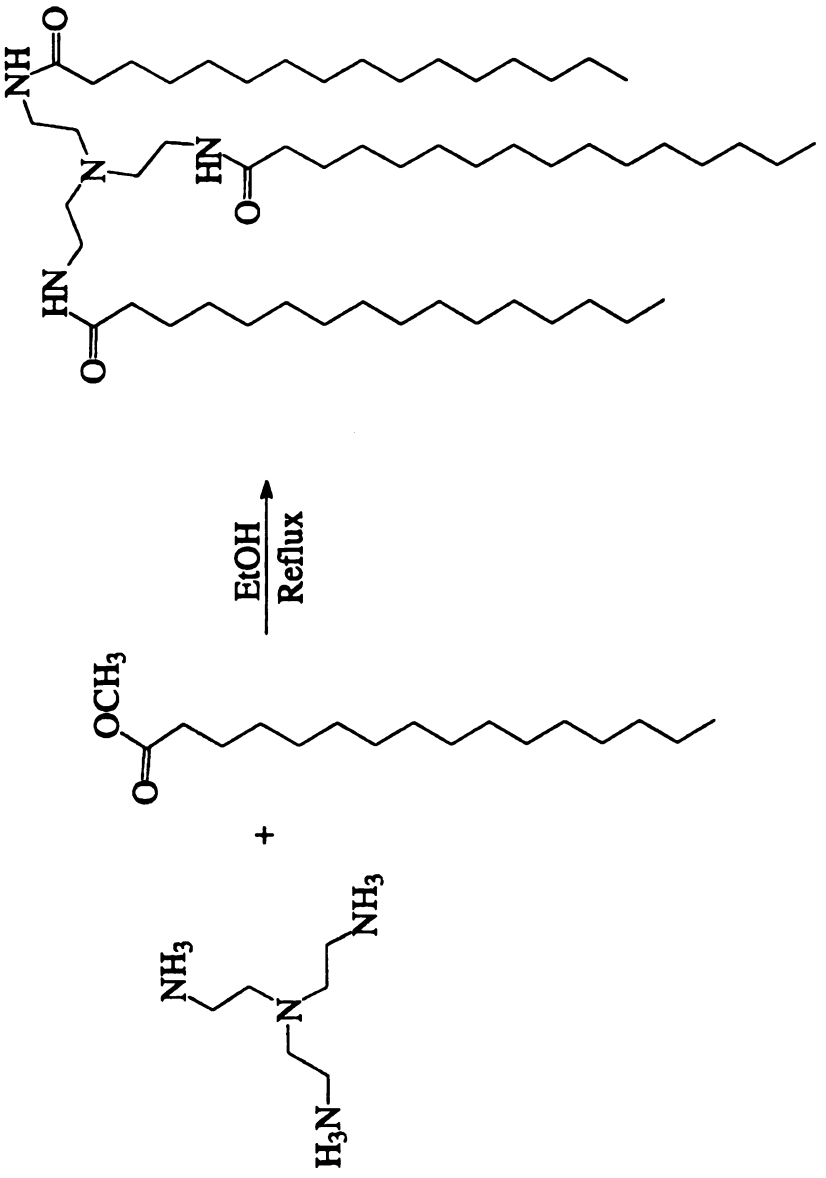


Figure A.1: Synthetic scheme for EI-3.

solid was washed several times with hexanes to remove excess fatty acid methyl esters. The product was purified by silica gel flash chromatography in 10:1 $CHCl_3$:methanol. Fractions were assayed by thin layer chromatography (TLC) on silica gel 60 in the same solvent system. Fractions that contained only EI-3 were pooled. The resulting pure product ran as a single spot on TLC (Figure A.2). The ^{1}H-NMR spectrum of the EI-3 shows the expected signals with trace levels of impurities detected. The assignments of the proton signals are given in Figure A.3, A. The small resonance at 3.4 ppm is due to deuterated methanol that was used along with $CDCl_3$ (7.24 ppm) to help completely dissolve the sample. Positive-ion electrospray ionization mass spectrometry (EI-MS) shows that the pure EI-3 is composed of a mixture of saturated fatty acids ranging from C14 to C18 (Figure A.3, B). The ion at 834.1 m/z corresponds to a protonated EI-3 that contains one C14 and two C16 fatty acids. The ion at 946.3 m/z corresponds to the protonated species containing three C18 fatty acids. From this spectra we deduced that the primary fatty acids are C16 and C18 with lesser amounts of C14, C15, and C17 present. This lipid is judged to be $\geq$ 98% pure.

The EI-4 synthetic scheme is shown in Figure A.4. Methanolysis of ethylenediaminetetraacetic acid (free acid) was performed in 5% HCl in methanol and refluxed for 4 hrs. The solvent was dried and the methylated product was dissolved in ethanol. Approximately 6 equivalents of 1-tetradecylamine were added and refluxed for 3 hrs. Solvent was removed by evaporation under reduced pressure and the product was washed several times with hexanes to remove excess 1-tetradecylamine. EI-4 was purified by silica gel flash chromatography with 100:10:0.2 $CHCl_3$:methanol:concentrated ammonium hydroxide as the solvent. Fractions were

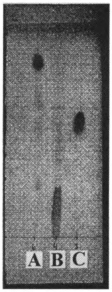


Figure A.2: Thin layer chromatography of EI-5 (lane A), EI-4 (lane B), and EI-3 (lane C). Solvent system was 10:1 chloroform:methanol and the plate was stained with phosphomolybdic acid (10%).

assayed by TLC in the same solvent system and the fractions that contained only EI-4 were pooled. TLC of the purified EI-4 gave nearly a single smeared spot with only trace levels of faster migrating components detected (Figure A.2). ^{1}H-NMR spectroscopy of EI-4 and the assignment of the proton signals are shown in Figure A.5, A. Only very low levels of impurities were detected. The signals at 3.35 ppm and 7.24 ppm are due to deuterated methanol and $CDCl_3$, respectively. Positive ion EI-MS (Figure A.5, B) shows the protonated molecular ion of EI-4 at 1073.9 m/z. The ion at 556.6 m/z corresponds to the doubly charged calcium adduct. The small peak at 214.8 m/z is due to protonated tetradecylamine that may arise from the fragmentation of EI-4 or may be a small impurity in the sample. The remaining cluster of ions from 437 m/z to 640 m/z could not be assigned. These ions may be due to multiply charge lipid aggregates or impurities in the sample. This lipid is judged to be $\geq$ 95% pure.

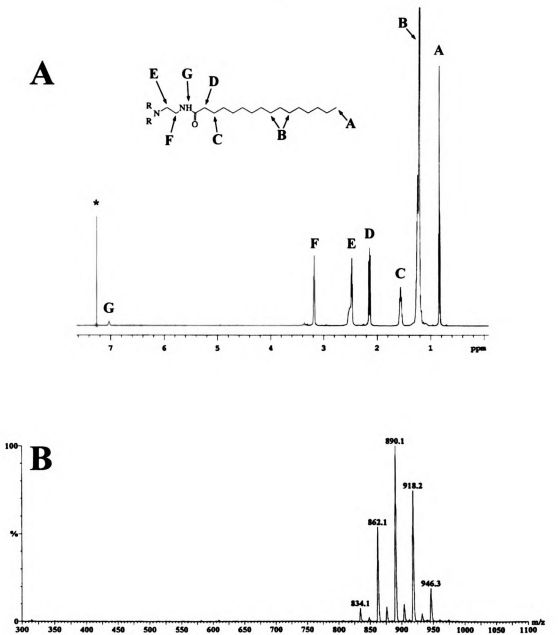


Figure A.3: Proton NMR spectrum (A) and positive-ion electrospray mass spectrum (B) of EI-3. NMR spectrum assignments are shown. Asterisks indicate solvent signals. Assignments of the mass spectrum are described in the text.

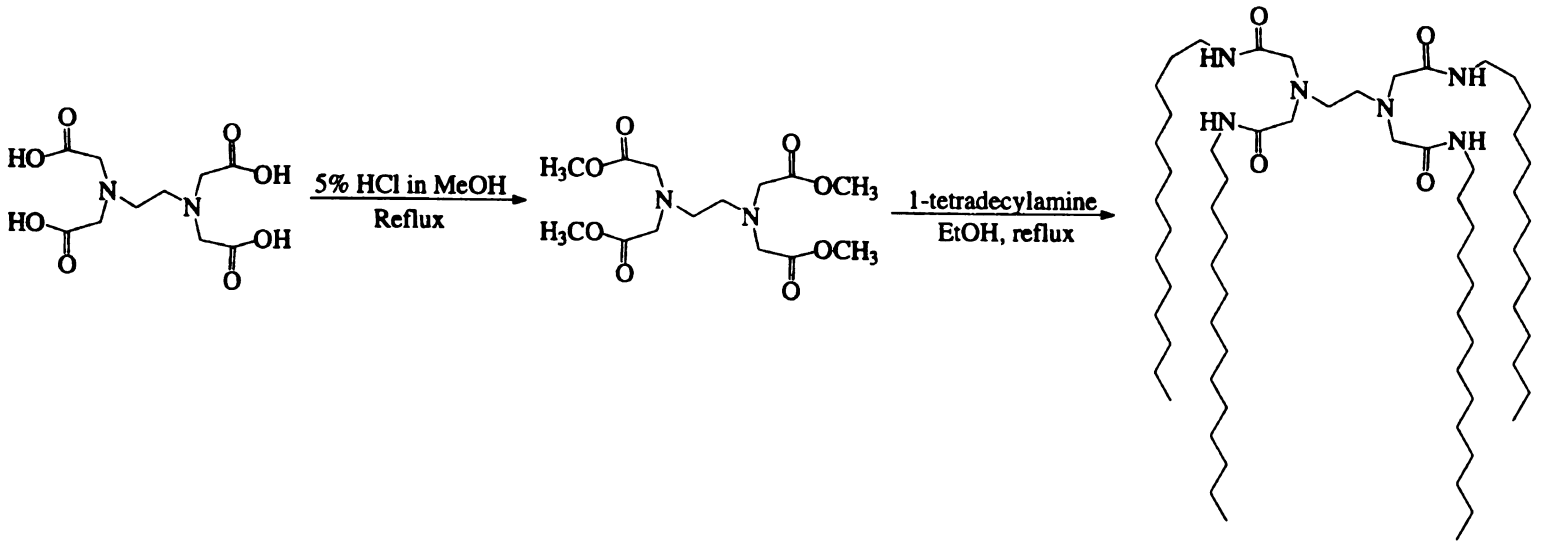


Figure A.4: Synthetic scheme for EI-4.

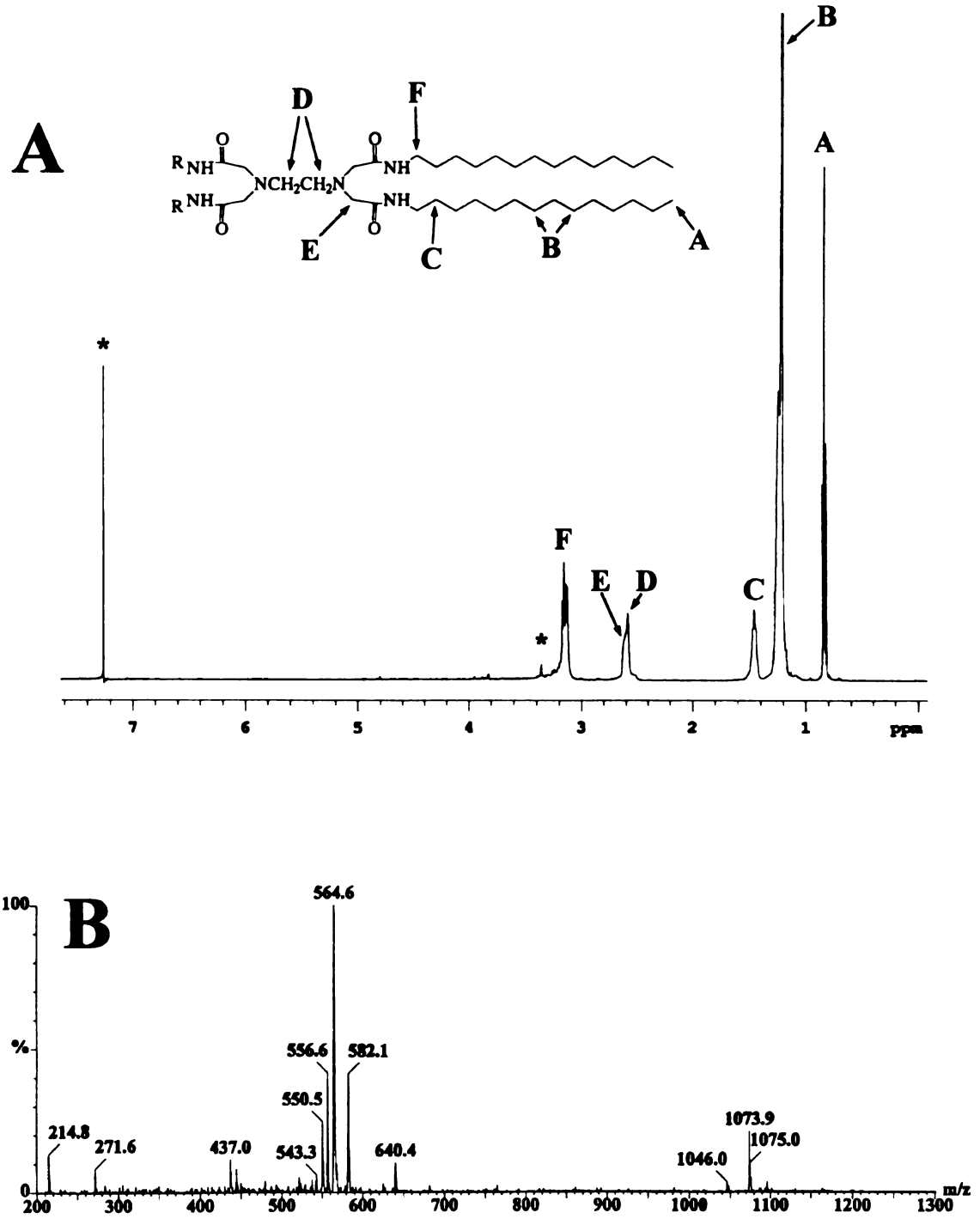


Figure A.5: Proton NMR spectrum (A) and positive-ion electrospray mass spectrum (B) of EI-4. NMR spectrum assignments are shown. Asterisks indicate solvent signals. Assignments of the mass spectrum are described in the text.

EI-5 was synthesized according to the scheme shown in Figure A.6. Tris(2-aminoethyl)amine was mixed with 4 equivalents of S-3-hydroxy-γ-butyrolactone. Nucleophilic attack of the primary amine on the carboxylic carbon opened the ring of the lactone to yield the corresponding aminolysis product. The viscous mixture was dissolved as completely as possible in 1:1 pyridine:acetonitrile and excess lauroyl chloride added. Addition of excess lauroryl chloride helped dissolve the reaction mixture to allow the reaction to proceed quickly. After one hour, the solvent was removed by evaporation under reduced pressure. Analysis of the reaction mixture by TLC showed at least eight components. Partial purification was accomplished by silica gel flash chromatography with two different solvents. Lauric acid was first eluted off the column with chloroform. The solvent was then changed to 98:2 chloroform:methanol to elute EI-5 that co-eluted with three other components. All of these components migrated very closely together on TLC. Preparative TLC in 10:1 chloroform:methanol was used for the final purification. The band corresponding to EI-5 was collected and product removed with 85:15 chloroform:methanol. Purified EI-5 migrated as a single major spot in TLC with only trace levels of slower migrating components present (Figure A.2). The ^{1}H-NMR spectrum of EI-5 and the assignments of the proton signals are shown in Figure A.7, A. Only trace amounts of impurities are detected. Positive-ion EI-MS spectrum shows the protonated molecular ion $(M+H^+)^+$ at 1546.8 m/z and the sodium adduct $(M+Na^+)^+$at 1568.8 m/z (Figure A.7, B). Assignment of the other ions are as follows: 785.1 m/z, $(M+H^++Na^+)^{2+}$; 795.9 m/z, $(M+2Na^+)^{2+}$; 811.9 m/z, $(M+2K^+)^{2+}$. The purity of EI-5 is judged to be ≥ 98%.

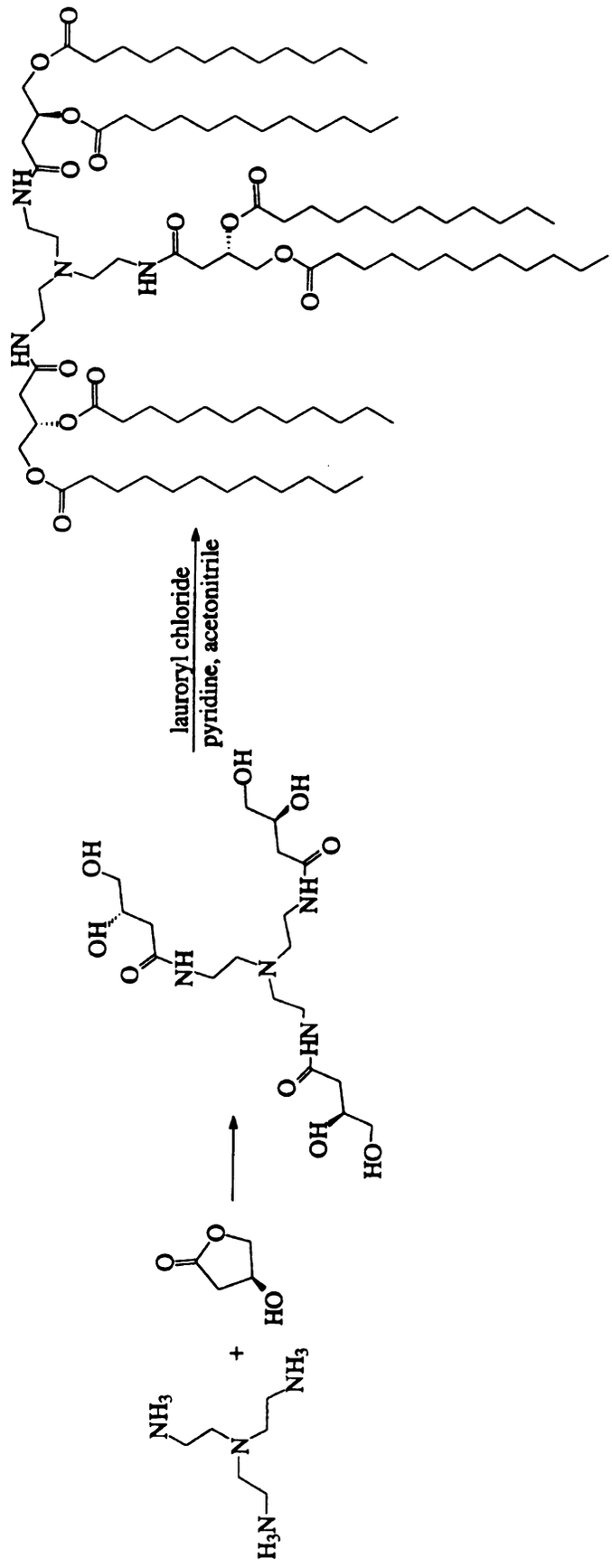


Figure A.6: Synthetic scheme for EI-5.

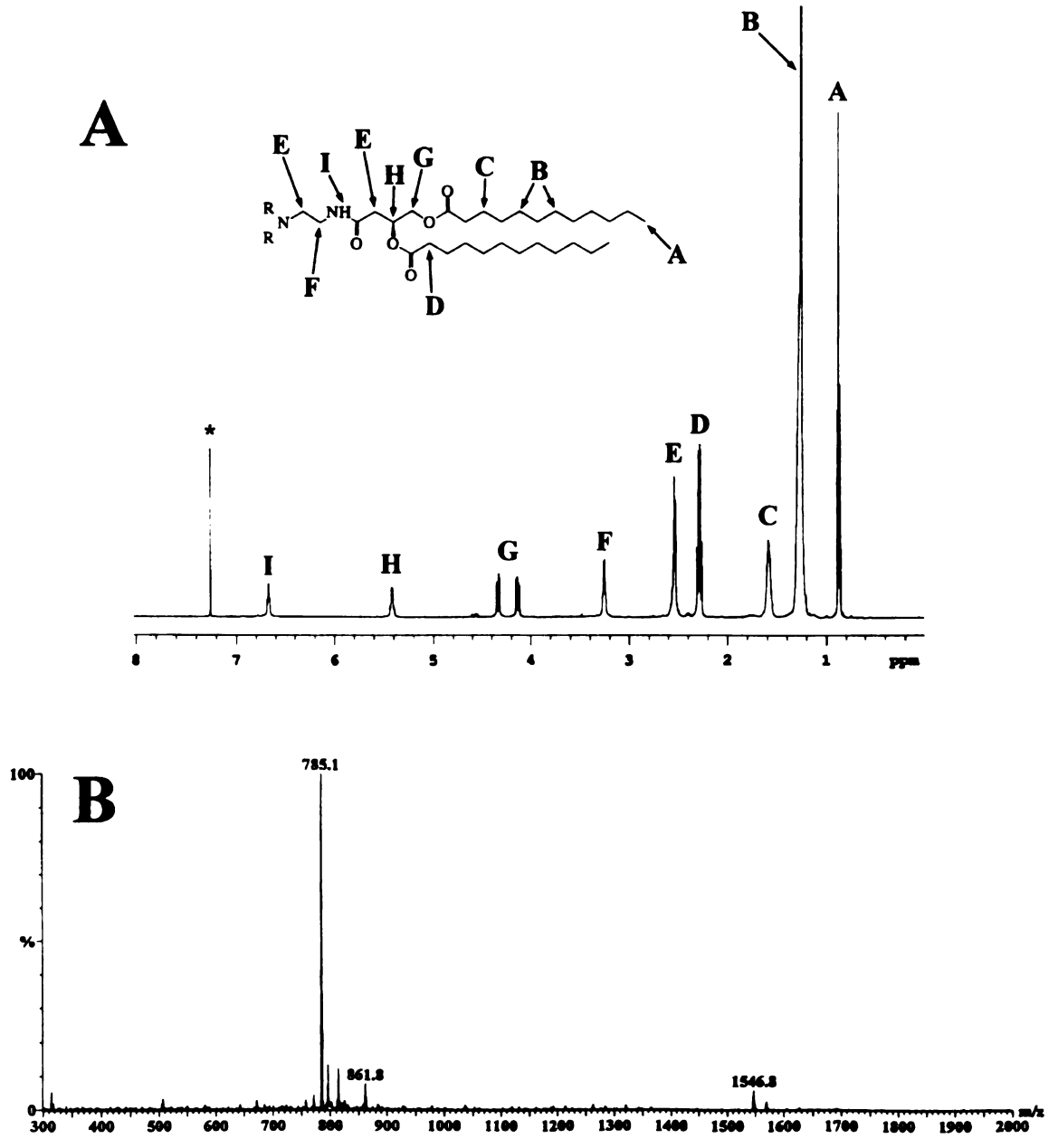


Figure A.7: Proton NMR spectrum (A) and positive-ion electrospray mass spectrum (B) of EI-5. NMR spectrum assignments are shown. Asterisks indicate solvent signals. Assignments of the mass spectrum are described in the text.

Characterization of the physical properties of EI-1, EI-2, EI-3, EI-4 and EI-5

In this report, I used x-ray diffraction, differential scanning calorimetry (DSC), and proton NMR T1 relaxation measurements to characterize the properties of the five lipids. This combination of techniques provides an extensive analysis of the dynamics and order of these lipids.

I observed the physical properties of the five synthetic lipids. Both EI-1 and EI-2 are waxy solids when dry and at room temperature. They readily hydrate when water is added to form a suspension. EI-3 and EI-4 were crystalline at room temperature. These lipids were very hydrophobic and were very resistant to forming a suspension in water. EI-5 is a waxy solid at room temperature. This lipid was also very hydrophobic and resistant to forming a suspension in water. Addition of an ethanol solution of EI-3, EI-4 or EI-5 to water was found to be the best way to obtain a fine aqueous suspension of the lipids. Vigorous sonication with a probe type sonicator worked to a lesser extent.

One of the most useful aspects of DSC is the ability to determine the temperature and characteristics of the lipid phase transitions. The width of the phase transition is correlated with the cooperativity of the transition. Sharp transitions indicate a large degree of intermolecular cooperation between the lipid molecules. The DSC scans from 5°C - 98°C of the five synthetic lipids are shown in Figure A.8. One of the lipids, EI-1, showed no detectable phase transitions in this temperature range. EI-2 experienced a fairly sharp phase transition at 74°C. Previous studies on this lipid indicate that the acyl chains of this lipid were not well ordered at 37°C (see Chapter 4). This is corroborated by x-ray diffraction data (shown below) which show that this lipid has fluid acyl chains at room temperature. Therefore, the observed phase transition is not a chain melting

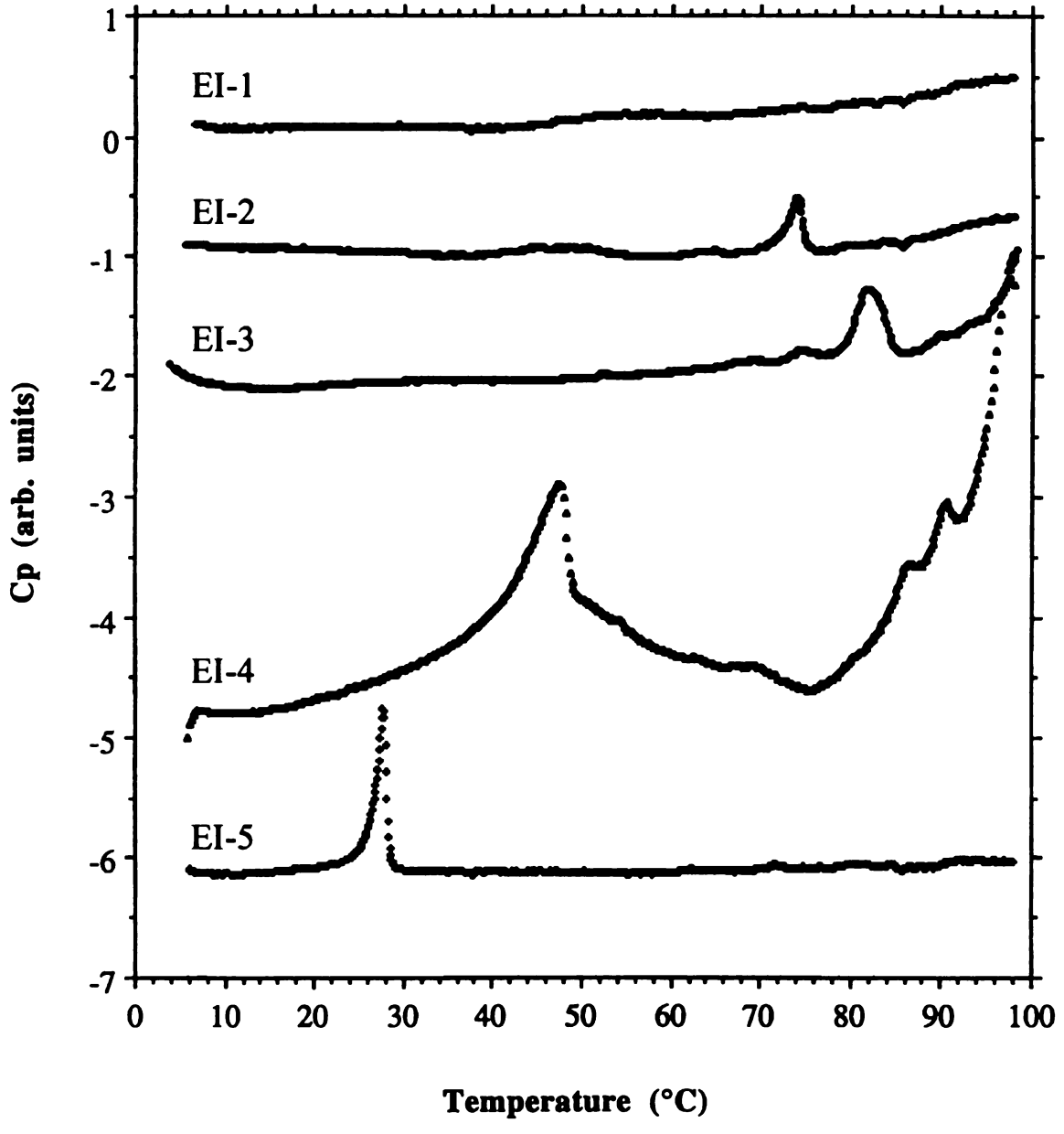


Figure A.8: Differential scanning calorimetry scans of the five synthetic lipids.

transition. EI-3 and EI-4 had more complex DSC traces and exhibited multiple high temperature phase transitions. EI-3 had a fairly broad phase transition at 82°C. The increase in the baseline at the end of the DSC trace (90°C-98°C) indicates the beginning of another phase transition at a higher temperature. EI-4 showed four distinct phase transitions at 47°C, 86°C, 91°C, and 97°C. EI-5 underwent a sharp phase transition at 27°C. These results indicate that these lipids, with the exception of EI-1, undergo cooperative phase transitions.

X-ray diffraction is a very powerful tool for studying the organization of lipids. In x-ray diffraction, only regular repeating planes of atoms (lattices) diffract the radiation. The angle of the reflection (diffracted x-rays) is governed by Bragg's Law:

$$n\lambda = 2d\sin\theta \qquad (1)$$

where n is the order of the reflection, λ is the wavelength of x-radiation, d is the spacing of the repeating unit, and θ is the angle of the incident and reflected radiation. Lipids typically have two types of repeating structures. The first is due to the bilayer repeat (large d) and gives low-angle reflections (small θ). The second is due to the packing of the acyl chains of the lipids (small d) and gives rise to wide-angle reflections (large θ). The wide-angle region is useful in determining the order of the acyl chains. Acyl chains which are rigid (all-*trans* conformation), such as found in gel phases, give sharp wide-angle reflections. The other extreme is found in acyl chains that have many *gauche* conformations and have a large degree of motional freedom (fluid-like phases). Acyl chains in this state give broad wide-angle reflections.

X-ray diffraction was used to study the molecular packing of the synthetic lipids. EI-3 had a well ordered x-ray diffraction spectrum (Figure A.9). The low angle region

contained five sharp reflections indicating a well ordered lattice. A sharp and intense chain packing reflection (4.16 Å) demonstrated that the acyl chains were rigid and very well ordered. EI-4 also gave several low angle reflections indicating a well-ordered lattice (Figure A.10). Three sharp reflections in the wide-angle region indicated three different chain packing distances (4.61 Å, 4.06 Å, and 3.74 Å). All of these reflections were sharp which indicated well ordered, rigid acyl chains. EI-5 gave two sharp low angle reflections (Figure A.11). The acyl chains gave three small, very sharp reflections superimposed on a broad reflection. The three sharp reflections would indicate a rigid acyl chain region while the broad reflection would indicate less ordered acyl chains typical of a fluid lipid phase. This may be explained by a coexistence of at least two phases, a phase with rigid acyl chains and a second with fluid acyl chains. These x-ray results indicate that all three of these synthetic lipids are well ordered. Both EI-1 and EI-2 show similar x-ray diffraction patterns when in water (Figures A.12 and A.13). These lipids had a very broad acyl chain packing reflection that is characteristic of lipid phases with fluid acyl chains. This demonstrates that EI-1 and EI-2 have a low degree of order in their acyl chains.

The methylene proton T1 relaxation times for the five synthetic lipids were measured as a function of temperature (Figure A.14). In these experiments, the magnetization is inverted (180° pulse) and allowed to recover to equilibrium. The recovery of the magnetization to equilibrium is exponential and is described by the equation:

$$M_\infty - M_t = 2M_\infty \exp(-t/T1) \qquad (2)$$

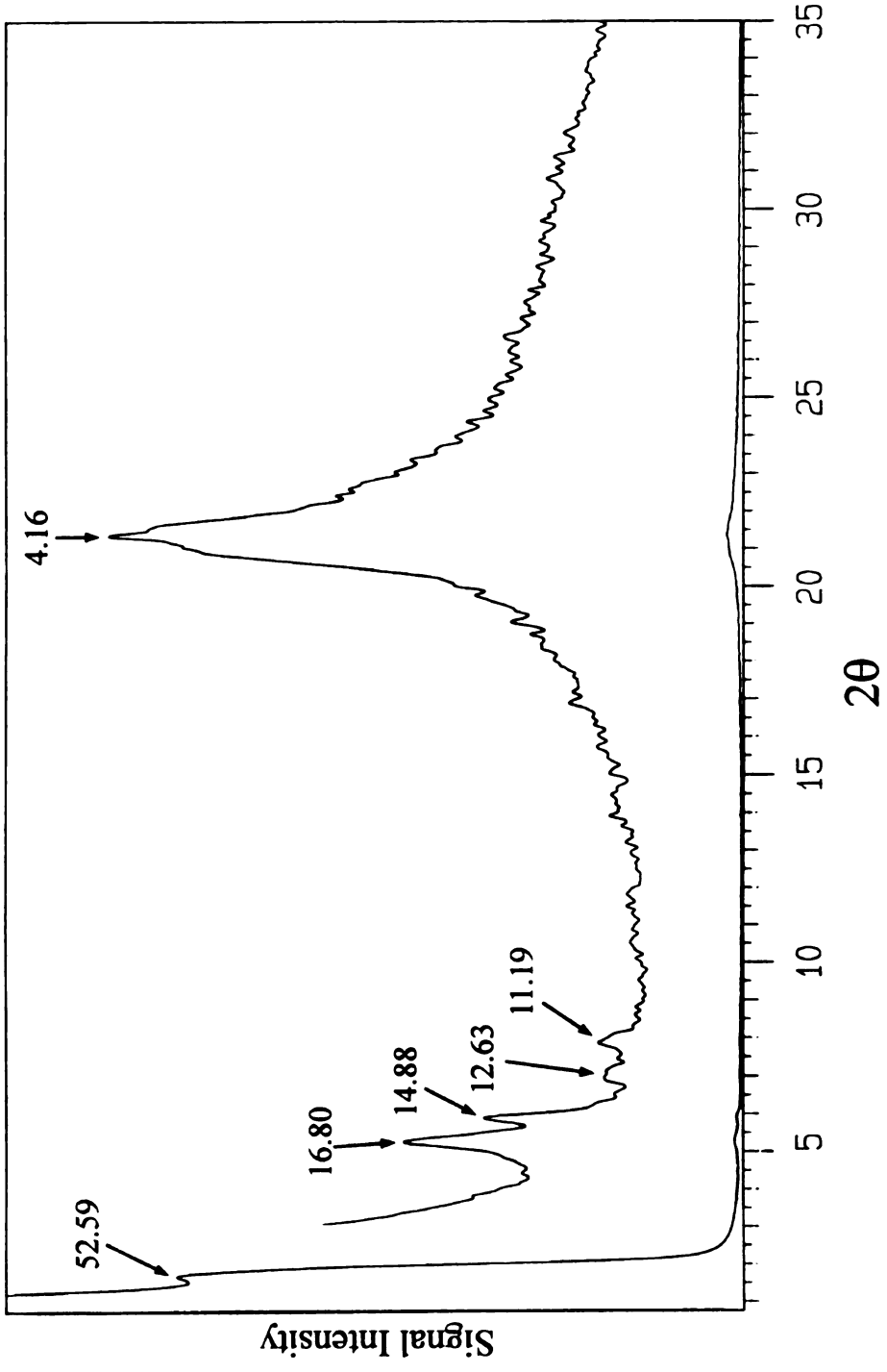


Figure A.9: X-ray diffraction profile of EI-3. Reflections are labeled in Angstroms.

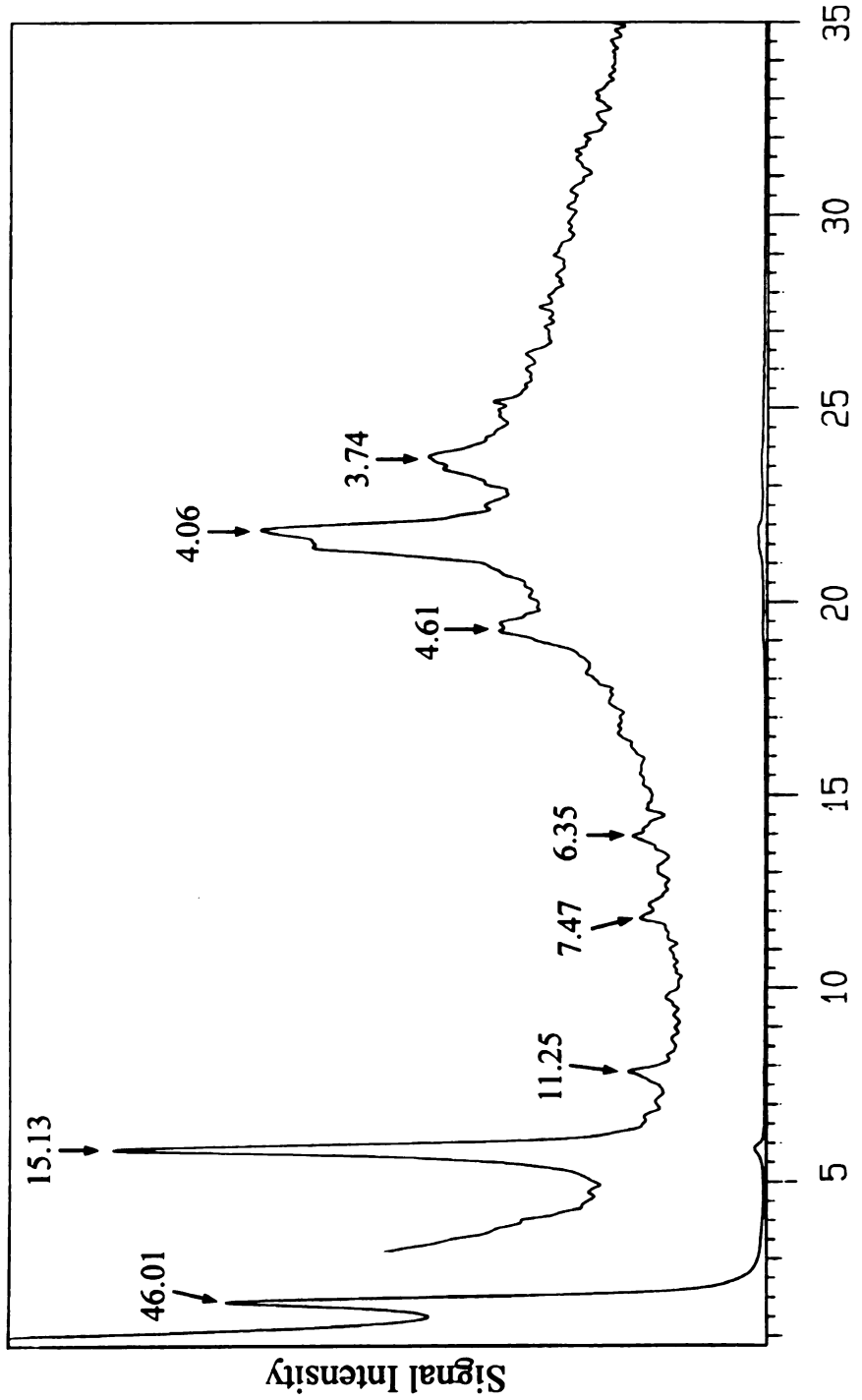


Figure A.10: X-ray diffraction profile of EI-4. Reflections are labeled in Angstroms.

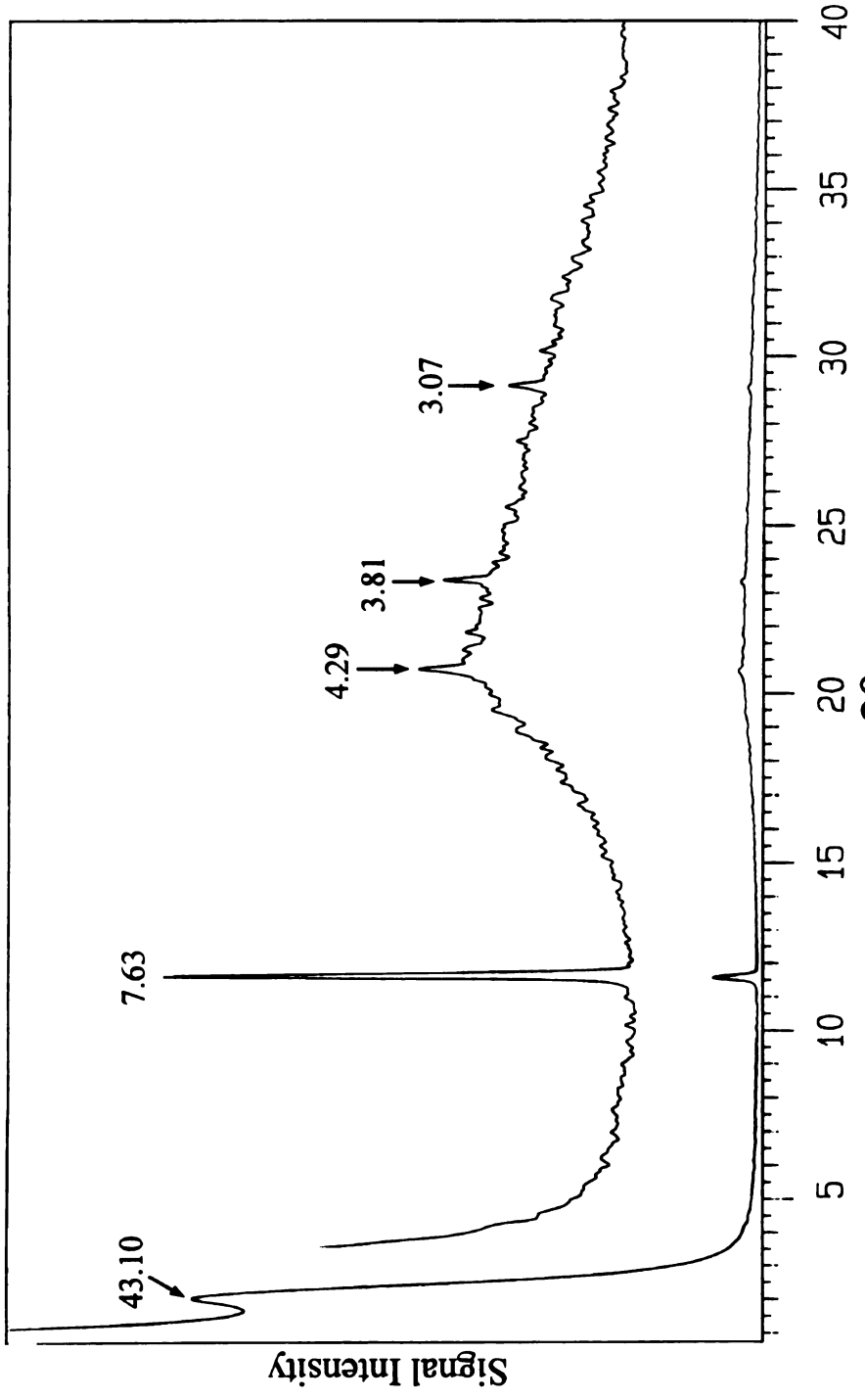


Figure A.11: X-ray diffraction profile of EI-5. Reflections are labeled in Angstroms.

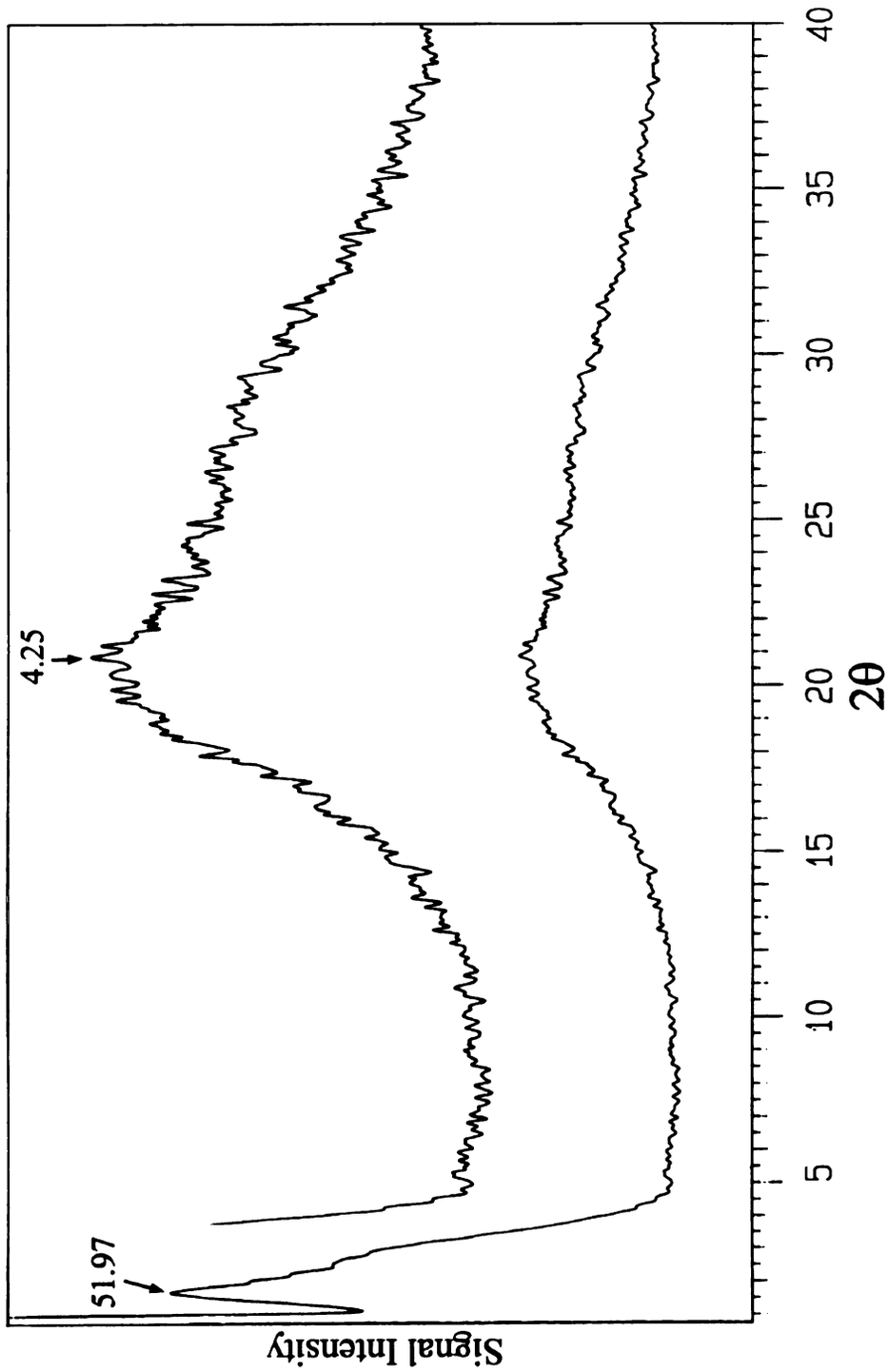


Figure A.12: X-ray diffraction profile of EI-1. Reflections are labeled in Angstroms.

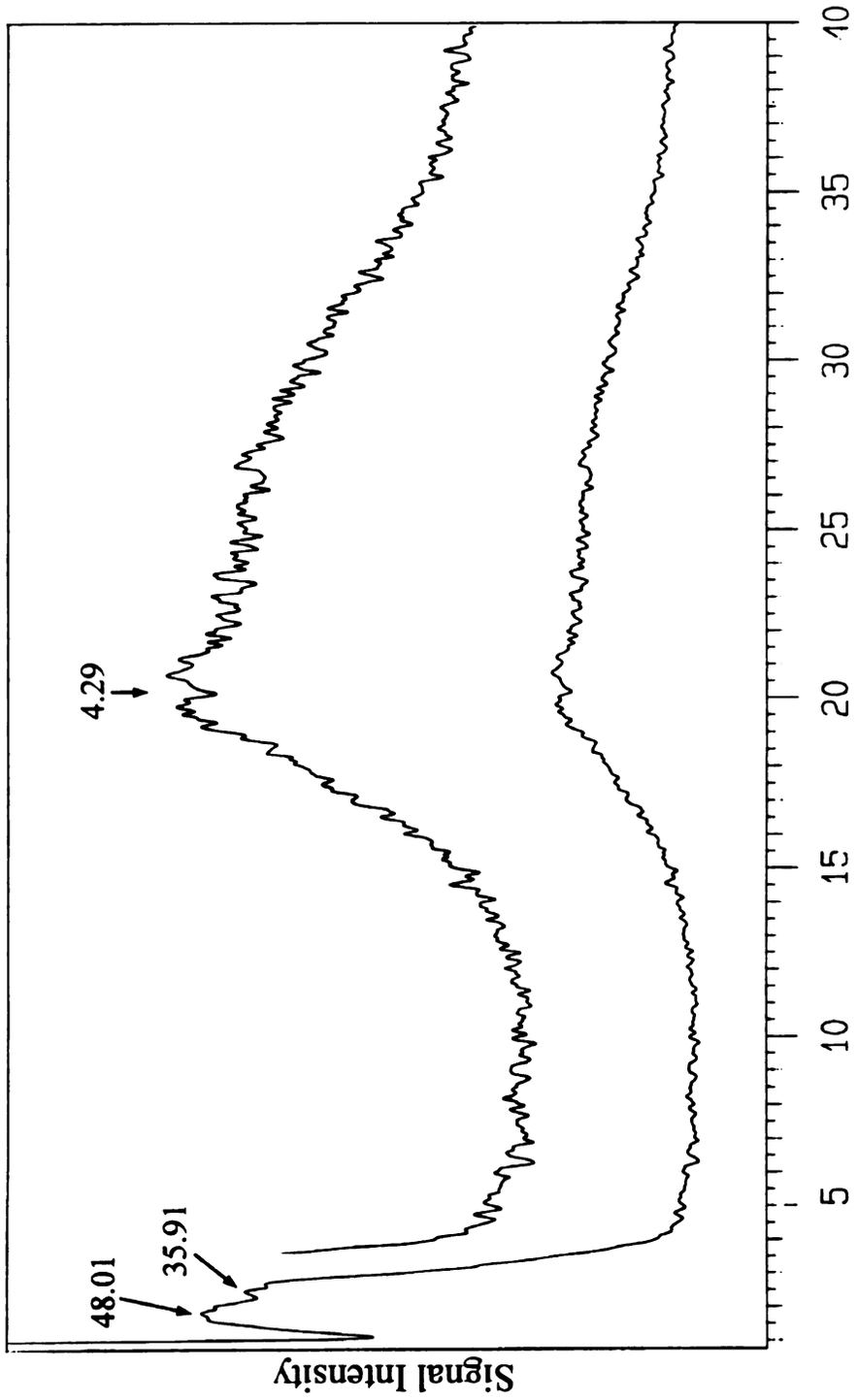


Figure A.13: X-ray diffraction profile of EI-2. Reflections are labeled in Angstroms.

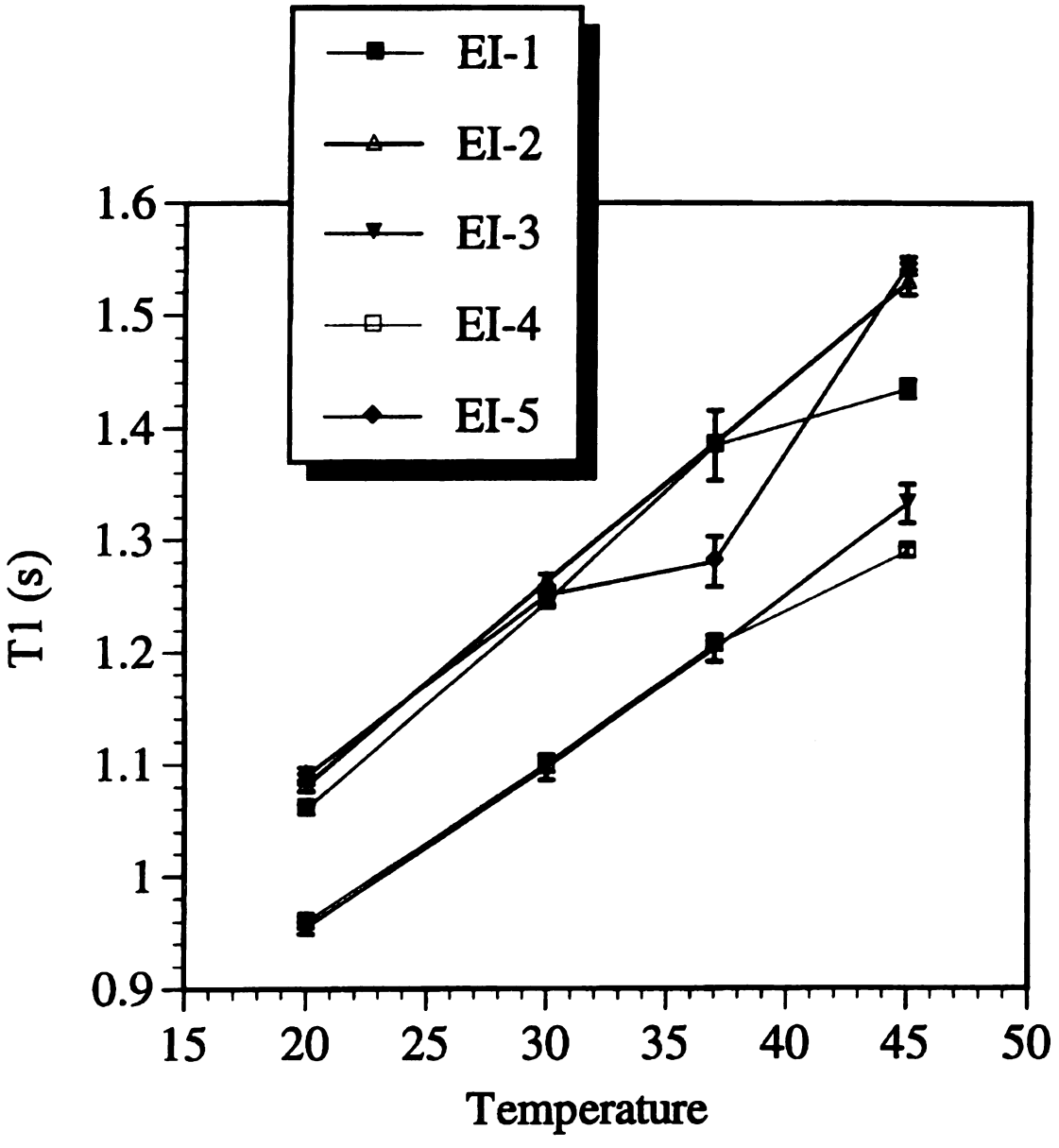


Figure A.14: T1 values for the methylene protons of the five synthetic lipids as a function of temperature.

where M_∞ and M_t are the magnetization at equilibrium and at time = t, t is the delay time and T1 is the spin-lattice relaxation time. For spin 1/2 nuclei, dipole-dipole interaction is the primary mechanism for relaxation (i.e., the interaction of a proton with another proton). T1 relaxation is dominated by the relaxation events occurring near the same rate as the NMR spectrometer frequency. For lipids at 300 MHz ($\sim 10^{-9}$ s), this corresponds to the *trans-gauche* isomerization of the carbon-carbon bonds in the acyl chains. Kroon et al. (173) have demonstrated that the T1 relaxation of lipid acyl chains are due to the intra-chain proton-proton interaction resulting from the *trans-gauche* rearrangement. Hence, our T1 relaxation times are measuring the rate of *trans-gauche* isomerization of the lipid acyl chains. For our experiments, the T1 relaxation is in the fast correlation region where an increase in *trans-gauche* isomerization rate results in an increase in T1. As expected, all of the lipids exhibited an increase in T1 (increase in the *trans-gauche* isomerization rate) with an increase in temperature. EI-1, EI-2, and EI-5 had the highest T1 values, indicating that their acyl chains have the fastest rate of motion. EI-3 and EI-4 had significantly lower T1 values indicating a lower rate of acyl chain motion in these lipids. This correlates well to the x-ray diffraction data. X-ray diffraction showed that EI-1, EI-2, and EI-5 had a large proportion of poorly ordered ('fluid') acyl chains. The x-ray diffraction patterns from EI-3 and EI-4 exhibited much sharper acyl chain reflections. This clearly demonstrates that these two lipids have a high degree of acyl chain order. The DSC data is also consistent with this result. We know that EI-1 and EI-2 do not undergo any phase transitions near or below room temperature and therefore, in conjunction with the x-ray diffraction data, shows that they exists in a fluid-like phase at least down to 5°C. EI-5 exhibits a sharp transition near room temperature (27°C) and

may be due to a chain 'melting' transition. EI-3 and EI-4, however, were much more stable and only had phase transition well above room temperature. This suggests that these lipids have a very rigid acyl chain region. It should be noted, however, that these NMR T1 measurements might not be sensitive to very rigid acyl chain motions due to dipolar coupling and chemical shift anisotropy (see Chapter 4). This could be a factor in EI-5 since x-ray diffraction indicated that some of its acyl chains might be highly ordered.

Conclusions

Our results clearly show that all five synthetic lipids form ordered systems typical of membranes. With the exception of EI-1, all of the lipids undergo concerted thermal transitions between 5°C and 98°C. According to x-ray diffraction, all of the lipids have regular repeating lattices. The x-ray diffraction and NMR T1 experiments indicate that EI-3 and EI-4 have well ordered, rigid acyl chains. The acyl chains of EI-1, EI-2, and EI-5 were less ordered and exhibited a higher rate of *trans-gauche* isomerizations than EI-3 or EI-4. However, it should be noted that EI-5 had some x-ray reflections in the wide-angle region that were very sharp. This indicates EI-5 may have a portion of its acyl chains highly ordered.

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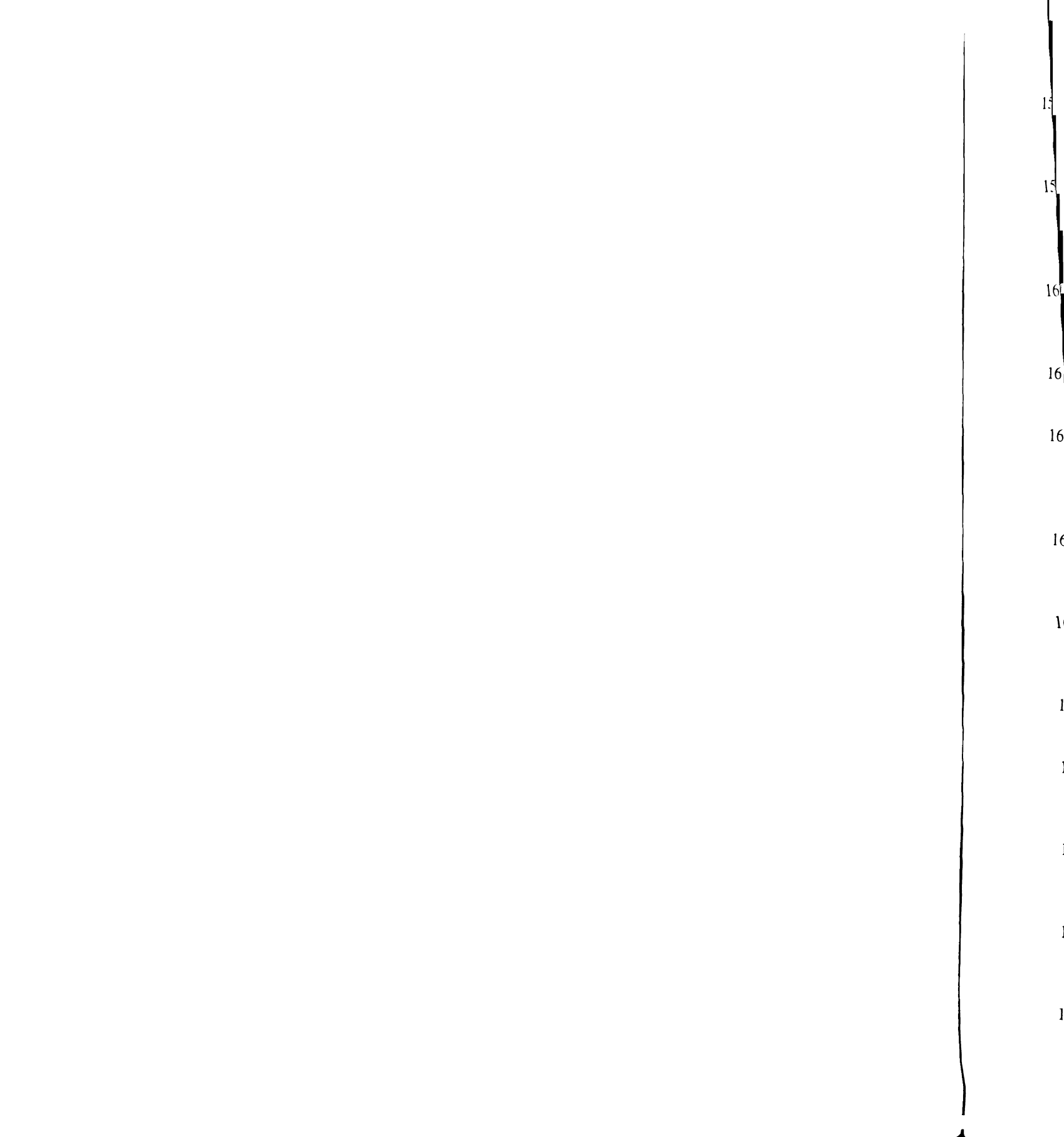

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