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19F-NMR STUDIES OF LIPID HYDROCARBON CHAIN ORIENTATIONAL
ORDER IN ACTINOLEPISMA LAIDLAWII B MEMBRANES

University — Université

UNIVERSITY OF ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Ph. D.

Year this degree conferred — Année d'obtention de ce grade

1984

Name of Supervisor — Nom du directeur de thèse

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¹³C-NMR Studies of Lipid Hydrocarbon Chain
Orientational Order in *Acholeplasma laidlawii* B Membranes

by

Peter Moore Macdonald



A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1984

THE UNIVERSITY OF ALBERTA

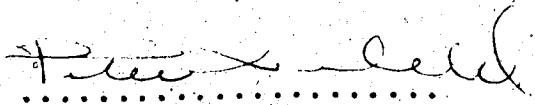
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Chain Orientational Order in
Acholeplasma laidlawii B Membranes
DEGREE FOR WHICH THESIS WAS PRESENTED Doctor of Philosophy
YEAR THIS DEGREE GRANTED September 1984

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled 'F-NMR Studies of Lipid Hydrocarbon Chain Orientational Order in *Acholeplasma laidlawii* B Membranes' submitted by Peter Moore Macdonald in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The hydrocarbon chain segmental orientational order parameters, S_{mol} , were obtained by fluorine-19 nuclear magnetic resonance spectroscopy using membranes of the organism *Acholeplasma laidlawii* B, whose complex lipids had been enriched with a variety of exogenous fatty acid and small amounts of particular isomeric monofluoropalmitic acid probes. The thermotropic behaviour of these membrane lipids was independently monitored using differential scanning calorimetry to permit an evaluation of the relationship between fatty acyl structure, orientational order and lipid phase state. First, a theoretical model of the fluorine spectrum of a monofluoropalmitic acid in a lipid bilayer was developed which permitted the spectral line shape to be interpreted in terms of an orientational order parameter, S_{mol} . The chemical shift anisotropy and dipolar interactions which dominate the fluorine spectra were ascribed a $\langle 3\cos^2\theta - 1 \rangle$ orientation dependence in both the gel and liquid-crystalline state as a result of axially symmetric lipid motions. The narrowing of the fluorine spectrum from its estimated effective chemical shift anisotropic and dipolar maximum was quantitated through the introduction of an orientational order parameter. Evaluations of the estimates and assumptions necessary to this type of analysis were developed as well as demonstrations of the correspondence of experimental and simulated fluorine spectra. Secondly, the values of S_{mol} were determined in the presence

of representatives from each class of naturally occurring fatty acid, including straight-chain saturated, mono-unsaturated, methyl branched and alicyclic ring substituted fatty acids, over a range of temperatures which spanned the gel to liquid-crystalline phase transition of the *A. laidlawii* B membrane lipids. In the liquid-crystalline state overall order was relatively low and the profile of order across the lipid bilayer was relatively independent of the "host" fatty acyl structure, always demonstrating a region of relatively constant order preceding a progressive decline in order towards the methyl terminus of the acyl chain. This uniformity of ordering permitted a characterization of the overall order in the liquid-crystalline state in terms of average forces such as proximity to the phase transition and temperature. In the gel-state, order increased profoundly and the presence of different fatty acyl structural substituents resulted in markedly different order profiles. The gel-state order profiles of straight-chain saturated fatty acids were almost flat, indicating that little head-to-tail gradient of order remained. In contrast, the order profiles in the presence of methyl *iso*- and *anteiso*-branched acids, or isomers of *cis*-octadecenoic acid with the double bond located near the methyl terminus, displayed large gradients of order in the gel state, indicative of the local disruptive influence of these structural substituents. When located near the center of the fatty acyl chain, structures such as the *cis*-double bond or cyclopropane ring

altered the gel state order profile so that order first decreased, then increased and then subsequently declined towards the methyl terminus. A *cis*-double bond located near the carbonyl headgroup had little disruptive influence on the gel state order profile. In addition to these local disruptive effects, specific structural substituents were capable of lowering the overall order in the gel state. When the dependences of order on temperature for individual cases of enrichments were normalized with respect to the lipid phase transition, it was observed that those fatty acyl groups giving the lowest phase transition temperature also exhibited the lowest degree of orientational ordering in the gel state. This suggested that different fatty acids modulate the temperature of the lipid phase transition by decreasing the stability of gel-state chain packing in both a local and a general sense. In the liquid-crystalline state overall order was *greater* in the presence of lower melting fatty acids following normalization with respect to the lipid phase transition. This effect was attributed to the overall temperature dependence of orientational order in the liquid-crystalline state rather than to some property of the particular fatty acid. When considered together these observations provided a unified picture of the manner in which fatty acyl chain structure modulates the lipid hydrocarbon chain orientational order and the temperature of the lipid phase transition.

ACKNOWLEDGEMENTS

The list of all those individuals who helped me to complete this study might require a separate volume. My thanks then to the multitude who, of necessity, must remain anonymous. Certain individuals, by virtue of their extensive contributions, require specific recognition and I would like to thank them here: Dr. Ronald McElhaney, my Supervisor, whose judgement, experience and perspective were as important as his support and encouragement to a young experimenter; Dr. Brian Sykes, for his infectious enthusiasm and for drawing back the veil which obscured the darker mysteries of magnetic resonance phenomena; Dr. Ruthven N.A.H. Lewis whose informal discussions carried me over several obstacles to the completion of these studies; Dr. Paul Cachia for aiding and abetting a novice organic chemist; Mr. Charles Seguin and Ms. Nanette Mak for their adept and enthusiastic technical assistance; Ms. Dawn Oare for transforming often illegible handwriting into readable material during the preparation of this thesis, and Mr. Perry d'Obrenan who prepared most of the figures contained herein. I would like to thank as well the Alberta Heritage Foundation for Medical Research for providing financial support during the course of this work. Finally, I want to thank my wife, Mary, for having faith.

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ABBREVIATIONS AND SYMBOLS USED IN THE TEXT

DGDG	Diglucosyl Diglyceride
DSC	Differential Scanning Calorimetry
FID	Free Induction Decay
GPDGDG	Glycerophosphoryl Diglucosyl Diglyceride
ΔH	Enthalpy Change
H_0	External Magnetic Field
Hz	Hertz
MFPA	Monofluoropalmitic Acid
MGDG	Monoglucosyl Diglyceride
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
OAPG	O-aminoacyl Phosphatidylglycerol
P.A.S.	Principal Axis System
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
ν_f	Radiofrequency
S_{CD}	Deuterium Order Parameter
S_{FF}	Difluoro Order Parameter
S_{mol}	Molecular Order Parameter
T_1	Longitudinal Relaxation Time
T_2	Transverse Relaxation Time
T_m	Lipid Phase Transition Temperature
T_r	Reduced Temperature

τ Motional Correlation Time
 ν Frequency (Hertz),
 $\Delta\nu$ Linewidth (Hertz)
 $\Delta\nu_q$ Quadrupolar Splitting
 ω Frequency (Radians-sec⁻¹)

1. INTRODUCTION

The earliest studies of biological membranes were concerned with delineating gross morphological features and generally conveyed an impression of a static structure. The description of the time-averaged or equilibrium structure of biological membranes is by no means complete and continues to be an important focus of contemporary research. However, it became apparent that biomembranes were essentially dynamic structures and that an understanding of structure-function relationships had to include an appreciation of the rates and amplitudes of the fluctuations about the "average" configuration.

Recognition of these considerations culminated in the proposal of the fluid-mosaic model of the structure of biological membranes by Singer and Nicholson (1972). The basic tenets of the model are now generally agreed upon and are as follows. Membranes consist mainly of lipids and proteins arranged in an amphiphilic lamellar structure with a relatively nonpolar inner layer separating two polar hydrated layers in contact with the external aqueous solution. The inner hydrophobic layer encompasses the hydrocarbon chains of the lipid molecules (arranged in a bilayer), the predominantly hydrophobic portions of the intrinsic membrane proteins as well as any molecules such as sterols or anaesthetics which preferentially partition into the membrane. The two surface layers consist of the lipid phosphate esters, carbonyls and headgroups, the carbohydrate

and hydrophilic amino acid residues of proteins as well as any associated ions and water. The amphipathic lipid bilayer acts to "solvate" the imbedded intrinsic proteins which may partially or entirely span the width of the membrane (for reviews, see Singer and Nicholson, 1972; Bretscher, 1973). This cooperative configuration can be considered an equilibrium structure since most membrane components will, of themselves, spontaneously aggregate or assume a lamellar structure when isolated and reconstituted in aqueous solutions (Bangham *et al.*, 1974).

Within the constraints of the planar membrane structure the individual components experience a considerable range of motional freedom. Intrinsic membrane proteins are more-or-less free to rotate (Cone, 1972) and diffuse laterally (Jain and White, 1977) within the plane of the bilayer. The membrane lipids can exist in either of two states, each of which differs markedly from the other in the degree of motional freedom permitted therein: a gel state, in which the lipid molecules are closely packed, experience restricted rotational or translational diffusion while the methylene chains assume an approximately all-*trans* configuration, and a liquid-crystalline state, in which lipid packing restrictions are greatly attenuated and where all degrees of motional freedom, including rotational and translational diffusion, rotational isomerization about individual bonds, and even trans-bilayer flip/flop of whole molecules, increase profoundly. The transition between these

two states can be triggered by changes in temperature (thermotropic), water content (lyotropic) or ion content (ionotropic). It is an approximately first order-phase transition and can be cooperative to the extent that hundreds of lipid molecules may melt simultaneously (Mabrey and Sturtevant, 1976; Lee, 1977). In biological membranes (as opposed to pure lipid-water systems) this lipid phase transition is always broader, the cooperativity being reduced by the presence of heterogeneities of lipid polar headgroup and hydrocarbon chain structure, or in some cases by the presence of sterols, hopanoid or related molecules.

The fluid-mosaic model was highly satisfactory, in that it embraced a wealth of experimental data within an elegantly simple hypothesis consistent with many of the properties of biological membranes. However, there remained a multitude of architectural and functional questions which were not immediately addressed. These issues included questions concerning: the function of the asymmetric distribution of lipid classes across the bilayer, the significance of lipid polymorphism such as the bilayer-to-hexagonal II transition, the role of the multitudinous structural variants present in the lipid headgroups and hydrocarbon chains, the manner and consequences of lipid-protein interactions, the molecular details of transport processes and of information transduction across membranes through receptors, the role of the cytoskeleton and its attachment to membrane proteins, protein dynamics

and their importance to bioactivity, and the as yet ill-defined role of membrane carbohydrates.

Perhaps the greatest single obstacle confronting the membrane biologist when attempting to approach the aforementioned issues is the fact that the usual principles and practices applicable to regular solution systems are impractical in the case of biological membranes. Biomembranes are solid and/or liquid-crystalline composites wherein the mutual interactions between membrane components are often of more significance than external solvent interactions. It is for this reason that spectroscopic techniques have figured so largely in the investigation of membrane phenomena where they are able to provide, variously, both structural and dynamic information. Membrane spectroscopic techniques may be conveniently classified into two categories: 1) those which depend upon the excitation of a naturally occurring intrinsic membrane component (i.e., infrared or laser Raman spectroscopy, nuclear magnetic resonance), and 2) methods which rely upon the signal arising from an extrinsic "reporter group" or "probe" (fluorescence; electron spin resonance). In fact, most spectroscopic methods *per se* have been applied in both senses.

Nuclear magnetic resonance (NMR) is one spectroscopic method capable of providing information on molecular structure and conformation as well as molecular motions and rate processes. A list of NMR-approachable membrane problems

culled from the literature would include studies of the orientation, mobility and lateral diffusion of fatty acyl chains as well as the conformation and dynamics of lipid headgroups, the investigation of transmembrane lipid flip/flop and of the interactions of small molecules such as cholesterol and anaesthetics with the lipid bilayer, the functioning of ion channels, of membrane fusion and fusogens, and more recently studies of protein-lipid interactions and the structure and dynamics of proteins in membranes (Jardetzky and Roberts, 1981; Jacobs and Oldfield, 1981).

At this point I would like to review the contribution of NMR to our understanding of the configuration and mobility of membrane fatty acyl chains, this topic being of singular relevance to the goals of the research to be described in this thesis.

¹H-NMR

Both sonicated and unsonicated lipid systems have been studied via ¹H-NMR and the spectra obtained in the two situations are qualitatively different. When gently dispersed in excess water, phosphatidylcholines (PC's) spontaneously form large multilamellar liposomes. With sonication small unilamellar vesicles can be formed. The differences in the resolution of the ¹H-NMR spectra between these two systems may be attributable to the vesicle size difference and the greater averaging of dipolar interactions

which accompanies the rapid tumbling of the smaller sonicated vesicles (Wennerstrom and Lindblom, 1977).

In the relatively high-resolution $^1\text{H-NMR}$ spectrum of a sonicated dispersion of PC in excess water, it is possible to assign many individual resonances such as the fatty acid methyl terminal, fatty acid methylene and choline N -methyl protons. Chapman (1968) was able to draw a number of important qualitative conclusions from the study of similar systems. It was apparent from the temperature dependence of the $^1\text{H-NMR}$ spectrum that the lipid bilayer existed in one of two states, a low temperature, quasi-crystalline state characterized by broad spectral lines (linewidth, $\Delta\nu > 10^3\text{ Hz}$) or a high temperature, quasi-liquid state characterized by much narrower spectral lines ($\Delta\nu < 100\text{ Hz}$). The transition between these two states occurred abruptly at a temperature which was dependent upon the chemical structure of the component lipids, tending to be lower with unsaturated lipids. It could be concluded as well that the lipids in the quasi-liquid state experienced an appreciable mobility reflected in the much narrower spectral lines observed therein. Furthermore, it was evident from the fact that the methyl resonances of the fatty acids were much narrower than the methylene resonances that there must exist a gradient of mobilities within the lipid bilayer.

The $^1\text{H-NMR}$ spectrum of an unsonicated dispersion of PC in excess water contains a single sharp central peak with unusually broad intense wings. This characteristic NMR line

shape is referred to as a superlorentzian. Since none of the individual component resonances are resolved the spectrum is difficult to analyze quantitatively. An interpretation of this line shape was presented by Wennerstrom and Lindblom (1977). The assumptions necessary to stimulate the superlorentzian are in themselves informative. It is sufficient to state here that this line shape can be obtained if it is assumed that overall vesicle tumbling is slow, that long-axis rotation of the lipid molecules is fast enough to provide effective cylindrical symmetry and reduce intermolecular interactions to near zero, and that the superlorentzian is a superposition of individual gaussian lines from lamellae assuming random orientations with respect to the magnetic field, the strength of the interaction scaling as $(3 \cos^2 \theta - 1)$, where θ is the angle between the magnetic field and the axis of cylindrical symmetry.

The low-temperature ¹H-NMR spectrum of unsonicated aqueous dispersions of PC shows a broad featureless signal which is no longer superlorentzian, indicating an absence of effective cylindrical symmetry (i.e., slow rotational diffusion) and a non-zero intermolecular interaction (i.e., slow lateral diffusion) (Chapman, 1968).

It is possible to obtain more quantitative information from the ¹H-NMR spectra of lipid dispersions by performing a moment analysis of the type reported by MacKay (1981) or by Kimmich *et al.* (1983). However, one is still restricted to conclusions regarding the overall motions of the lipid.

molecules rather than being able to selectively probe particular locales. Briefly, the moment analysis measures the extent of molecular motions by comparing the experimental spectral moment for a particular situation (M_{nr}) with that calculated for the rigid-lattice case. Since molecular motions will tend to average the parameters of interaction, the experimental or residual moment will be reduced from the calculated rigid-lattice moment in proportion to the type and extent of particular motional modes. McKay (1981) was able to resolve the second moments (M_{2r}) of the hydrocarbon chains and headgroups of DPPC by a comparison of DPPC vs chain-perdeuterated DPPC. The value of M_{2r} for the hydrocarbon chains was observed to decrease by more than an order of magnitude over the temperature range -30 to 55°C with two sharp drops: one at the DPPC pre-transition temperature of 35.5°C, and the other at the main transition temperature of 41°C. It was possible to conclude that even in the low temperature quasi-crystalline state the lipid molecules experience a considerable degree of motional freedom, probably due to molecular rotations and rotational isomerizations of the methylene chains.

¹³C-NMR

Considerably more detailed information on the molecular motions present in the lipid components of membranes has been obtained via ¹³C-NMR using either natural abundance or specific enrichment techniques. It is therefore possible to

observe and characterize the ^{13}C resonance line for each and every carbon atom in the lipid molecule. In particular, it was clearly evident even in the first ^{13}C measurements on sonicated dispersions of DPPC that a gradient of longitudinal relaxation times (T_1) existed from the carbonyl headgroup of the fatty acid chain towards the methyl terminus (Metcalf *et al.*, 1971; Levine *et al.*, 1972). The spin-lattice relaxation time, T_1 , quantitates the rate at which the z component of the net magnetization (that component aligned perpendicular to the external magnetic field in a 3-dimensional coordinate system) returns to its equilibrium value following excitation by a radio frequency (r_f) pulse. The relaxation occurs through an interaction with local oscillating magnetic fields which are present in the nucleus' immediate surroundings or lattice. Relaxation mechanisms depend on molecular motions and relaxation measurements can thus provide an insight into these motions.

When discussing molecular motions it is important to distinguish the properties of order and mobility (Seelig, 1977). The concept of molecular order refers to the geometric constraints on the amplitude of particular motions, while mobility refers to the rates at which particular motions occur. The two are not necessarily interdependent in that it is possible to observe instances in which high order is associated with greater mobility and *vice versa*. The gradient of relaxation times observed by ^{13}C -NMR must be considered to reflect both order and

mobility effects, the difficulty being to distinguish the two. If the rates of motion are constant along the length of the acyl chain, then amplitudes must increase towards the methyl terminus and, similarly, if order is constant then mobility must increase towards the chain ends. In fact, as discussed by Lee *et al.* (1976), either of these models or a hybrid, in which both order and mobility change, could account for the ^{13}C relaxation data.

^2H -NMR

A greater distinction between order and mobility is possible using ^2H -NMR techniques. When specific protons are replaced by deuterons it is possible to characterize both the order and mobility at specific locations in the lipid molecule in a nonperturbing fashion. For these reasons, ^2H -NMR has proven to be the most powerful and productive approach applied to membrane lipids.

In an unoriented sample (i.e., in most membrane preparations), the deuterium quadrupole interactions which dominate the spectrum give rise to a characteristic powder pattern. The spectrum has two peaks, the separation of which is termed the deuterium quadrupole splitting, $\Delta\nu_q$, which is in turn used to calculate the deuterium order parameter, S_{CD} , according to

$$\Delta\nu_q = (3/4)(e^2qQ/h)S_{\text{CD}} \quad [1]$$

where e^2qQ/h is the static deuterium coupling constant (Seelig, 1977; Seelig and Seelig, 1980). S_{CD} is a measure of the motional anisotropy of the C-D bond and is related to the average amplitude of motion by the equation:

$$S_{CD} = \overline{1/2(3\cos^2\beta - 1)} \quad [2]$$

where β is the angle between the C-D bond vector and the direction of the bilayer normal and the bar denotes the time average. Measurements of S_{CD} provide structural or configurational information. Deuterium NMR relaxation time measurements shed light on the dynamics of the lipid molecules, permitting a distinction between order and mobility. However, the slow motion effects manifest in the 2H -NMR spectra of gel-state lipids do not permit the application of an useful order parameter formalism in the gel state (Davis, 1983) and this is one significant shortcoming of 2H -NMR.

When DPPC was deuterio-labelled in both fatty acyl chains at the C-2 segment next to the carbonyls, the 2H -NMR spectrum showed three doublets of quite different separation. By labelling the chains individually the largest signal could be assigned to the *sn*-1 chain while the two smaller signals were assigned to the *sn*-2 chain (Seelig and Seelig, 1975). It was concluded that the two chains of DPPC were physically inequivalent and adopted different average conformations in the liquid-crystalline bilayer. In fact, similar splitting behaviour has been observed at the C-2 segment for phospholipid bilayers composed of different

headgroups (choline, ethanolamine, serine) and fatty acids (saturated and unsaturated) (Seelig and Browning, 1978), as well as in natural membranes enriched with C-2 deuterated fatty acids such as *Escherichia coli* (Gally *et al.*, 1979) and *Acholeplasma laidlawii* B (Stockton *et al.*, 1977; Rance *et al.*, 1980).

The underlying molecular picture was revealed through a quantitative analysis of these quadrupole splittings (Seelig and Seelig, 1975; Schindler and Seelig, 1975). It could be concluded that the *sn*-1 chain is extended perpendicular to the bilayer surface at all segments while the first CH₂ segment of the *sn*-2 chain is oriented parallel to the surface of the membrane, the chain subsequently bending sharply to permit a perpendicular orientation of the remaining methylene segments. These conclusions were confirmed by neutron diffraction (Zaccai *et al.*, 1979; Buldt and Seelig, 1980) and X-ray structural studies (Hitchcock *et al.*, 1974; Pearson and Pascher, 1979). Thus, it would appear that the configurational peculiarity of the *sn*-2 chain is an ubiquitous phenomena independent of the physical state of the lipid and typical of both model and biological membranes.

A quantitative characterization of the local orientational order along the entire length of the acyl chain is possible with ²H-NMR by selectively labelling each segment of the hydrocarbon chain and measuring the order parameter, S_{CD}, of the individual segments. The measured

value of S_{CD} can be related to the molecular order parameter S_{m01} , according to:

$$S_{m01} = -2 S_{CD} \quad [3]$$

The molecular order parameter is intended to quantitate the amplitude of the average fluctuations of the acyl chain about an axis perpendicular to the plane of the bilayer (i.e., parallel to the bilayer normal). If the acyl chains are fixed in an all-*trans* configuration aligned parallel to the bilayer normal and rotating only about their long axis, then $S_{m01} = 1$. At the other extreme of completely statistical movement through all possible angles, then $S_{m01} = 0$. For extensive discussions of these concepts, see Seelig (1977) and Seelig and Seelig (1980).

The order profile of the lipid bilayer (that is, the variation of the order parameter, S_{m01} , with the position of the segment in the chain) has come to be recognized as the "signature" of the lipid bilayer. The order profile of DPPC in the liquid-crystalline state is characteristic in that values of S_{m01} are relatively constant from carbon atoms 2-9 and thereafter decrease towards the methyl terminal. Thus the methylene segments near the end of the chain experience far greater amplitudes of angular fluctuation than those closer to the carbonyl headgroup (Seelig and Seelig, 1974). It may be further concluded that, although the hydrocarbon interior of the bilayer is liquid-like in the liquid-

crystalline state, there is still a considerable degree of organization and ordering present, since values of S_{mol} average approximately 0.35. Of course, very small average values of the order parameter would be inconsistent with a bilayer structure.

It is interesting to note that beyond the C-4 segment, only a single quadrupole splitting is observed per methylene segment even when both fatty acyl chains are deuterio-labelled, indicating that the two chains are approximately motionally equivalent over most of their length.

The considerable effort required to synthesize selectively deuterated phospholipids has restricted the range of different lipid structures investigated to date. Nevertheless a representative collection of different lipid classes can be assembled. These include PC's containing various fatty acyl substituents such as dipalmitoyl (Seelig and Seelig, 1974, 1975), dimyristoyl (Oldfield *et al.*, 1978 a,b), palmitoyl-oleoyl (Seelig and Seelig, 1977; Seelig and Waespe-Sarčević, 1978), palmitoyl-elaidoyl (Seelig and Waespe-Sarčević, 1978) and di-dihydrosterculoyl (Dufourc *et al.*, 1983), as well as dipalmitoyl phosphatidyl serine (DPPS) (Seelig and Browning, 1978; Browning and Seelig, 1980), dimyristoyl phosphatidylethanolamine (DMPE) (Marsh *et al.*, 1983) and a glycolipid (Skarjune and Oldfield, 1979). To a first approximation it can be stated that the order profiles observed in each of the bilayers composed of these various lipids were similar, showing an initial

plateau region of relatively constant order preceding a progressive decrease in order toward the methyl terminal.

In order to permit a direct comparison between a variety of lipid structures which differ widely in the physical state that they assume at a given temperature, it is necessary to refer to some common corresponding state. This is achieved by comparing the order profiles at a reduced temperature, T_r , such that

$$T_r = (T - T_m) / T_m \quad [4]$$

where T is the measuring temperature and T_m is the temperature of the particular gel to liquid-crystalline phase transition. When such a comparison was made by Seelig and Browning (1978) it was immediately apparent that, despite large differences in chain and headgroup chemistry and regardless of the absolute temperature of the main lipid phase transition, the overall order and the order profiles were very similar in each of these diverse lipid structures, provided they were compared at identical values of the reduced temperature.

These similarities in the order profile are carried over from model membranes to biological membranes. *E. coli* membranes enriched with deuterated palmitic or oleic acid (Gally *et al.*, 1979), as well as *A. laidlawii* B membranes enriched with deuterated myristic (Jarrell *et al.*, 1982), palmitic (Stockton *et al.*, 1977), oleic (Rance *et al.*, 1980)

or dihydrosterculic (Jarrell *et al.*, 1983) fatty acids, have been characterized by $^1\text{H-NMR}$. When the order profiles from these biological membranes are compared at a reduced temperature they are nearly identical to those of the synthetic model membranes.

While the similarities between the order profiles obtained under such a remarkably diverse set of conditions are striking, the differences in order are worthy of note. The order profile itself is subject to some variation due to the presence of specific structural substituents within the fatty acid chain. When the order profile of deuterated palmitic acid incorporated into POPC was compared with DPPC it was apparent that the presence of a *cis*-double bond had induced a local stiffening in the adjacent palmitic acid chain reflected in an increased order parameter in the region C-5 to C-9 (Seelig and Seelig, 1977). Furthermore, an overall ordering effect of *cis*-unsaturation was evident along the entire length of the neighbouring palmitate chain.

The order profile of the oleic acid chain itself was quite unusual, showing a pronounced dip or discontinuity at the location of the *cis*-double bond (Seelig and Waespe-Sarčević, 1978). A quantitative analysis showed that the dip in the S_{CD} profile was attributable to a tilt of the C=C bond vector of $7-8^\circ$ with respect to the bilayer normal. After correction for this geometric factor the molecular order parameters, $S_{\text{m}01}$, were identical to those observed in deuterated DPPC. This geometric consideration applies to the

orientation of the *CIS*-double bond in model systems, as described above; as well as in biological membranes since the same dip in the S_{CD} order profile at the location of the double bond was observed in membranes from *E. coli* (Gally *et al.*, 1979) or *A. laidlawii* B (Rance *et al.*, 1980) enriched with deuterated oleic acids.

An analogous geometric consideration was invoked to interpret the deuterium splittings observed with deuterated dihydrosterculic acid incorporated either synthetically into PC's (Dufourc *et al.*, 1983) or biosynthetically into membrane lipids of *A. laidlawii* B (Jarrell *et al.*, 1983). It was concluded that the C9-C10 vector of the cyclopropane ring-containing segment of 19:0cp,c Δ ' was tilted at 89° with respect to the bilayer normal. However, unlike the case of the *CIS*-double bond, when corrected for this geometric consideration the values of the molecular order parameter, S_{m01} , in the vicinity of the cyclopropyl substituent were substantially greater than those obtained in the case of a straight-chain saturated fatty acid. It was, therefore, concluded that the cyclopropane ring acts as a boundary between two regions of the fatty acyl chain within the lipid bilayer: one region (between the carbonyl headgroup and the cyclopropane ring) having restricted amplitudes of motion and a second (between the cyclopropane ring and the fatty acyl methyl terminus) having a much greater degree of motional freedom.

It is most interesting that the configurational and motional details of the fatty acyl chains elucidated via $^2\text{H-NMR}$ in model systems have been in each case confirmed as occurring as well in biological systems such as *E. coli* or *A. laidlawii* B membranes. Whether it be the unique configuration of the C-2 segment of the *sn*-2 esterified fatty acid, the shape of the order profile, the orientation of a *cis*-double bond or of a *cis*-cyclopropyl ring substituent, each phenomenon was observed in both biological and model membranes. It must be concluded that the orientational chain order of the phospholipids is not extensively perturbed by the presence of membrane proteins.

Although it has been noted earlier that the order profile is characteristic of fatty acyl chain configurations, the overall order, that is the chain-average orientational order, is subject to some secondary variation as the structure of the lipid headgroup is altered. For example, Seelig and Browning (1978) compared the overall order observed in bilayers composed of DPPC with that of DPPS. When compared at a common reduced temperature the overall order of the DPPS bilayers was greater than that of the DPPC bilayers. In a similar study, Marsh *et al.* (1983) discerned that lamellae composed of DMPE were overall more ordered than those composed of DMPC when the data was normalized with respect to the phase transition temperature. To put these observations in their proper perspective, it should be noted that the overall order of the palmitoyl

chain of POPC was greater than either DPPS or DPPC (Seelig and Browning, 1978) so that the presence of a single *cis*-double bond more profoundly affected the overall order than, for example, the introduction of a net negative charge in the polar headgroup.

In addition to measurements of the quadrupolar splittings, $\Delta\nu_Q$, which furnish information about the average amplitude of the angular fluctuations undergone by the hydrocarbon chains, $^2\text{H-NMR}$ relaxation time measurements can provide information on the rates of segmental motion. Such measurements have been performed using selectively deuterated DPPC (Brown *et al.*, 1979), on DPPC containing perdeuterated fatty acyl chains (Davis, 1979) or selectively deuterated stearic acids incorporated into egg PC vesicles (Kton *et al.*, 1976).

The dependence of the deuterium relaxation time, T_1 , on segment position revealed a number of interesting trends also observed via $^{13}\text{C-NMR}$ relaxation measurements as described earlier. The glycerol backbone had the shortest relaxation time, implying that this portion of the molecule moves the slowest. In contrast, the longest relaxation times were observed for the methyl groups located at the fatty acyl chain terminus and quaternary nitrogen, indicating very fast internal rotations of these groups. The relaxation times of the two methylene segments in the headgroup of DPPC (or of DPPE or DPPS) were very similar, indicating a strongly correlated motion of these two segments. The

relaxation times of the methylene segments of the fatty acyl chains were approximately constant over the region C-3 to C-9 and subsequently increased progressively toward the methyl terminus, indicative of a gradient of the rates of motion toward the center of this bilayer.

Ultimately it is desirable to express the rates of these motions in terms of a correlation time, τ_C , for a particular motion. The following simplifications have generally been employed when converting deuterium relaxation times into correlation times. Since all T_1 values increase with increasing temperature, it is assumed that the correlation time falls into the so-called short correlation time regime with $\omega_0^2 \tau_C^2 \ll 1$, where ω_0 is the measuring frequency in rad/sec. It is further assumed that the motions responsible for the relaxation can be described by a single correlation time, τ_C . If these assumptions hold then the following expression relates the relaxation time, the order parameter and the correlation time (Seelig, 1977; Davis *et al.*, 1978):

$$\frac{1}{T_1} = \frac{3}{8} \left(\frac{e^2 g_Q}{h} \right) (1 - S_{CD}^2) \tau_C \quad [5]$$

As with the ^{13}C relaxation measurements, the deuterium T_1 relaxation times depend on both the ordering and rate of motion. The measurement of both deuterium order

parameters and relaxation times thus permits a distinction between rate and amplitude or mobility and order. When the motional correlation times were derived using the above equation and compared with the order parameters as a function of segment position, the shapes of the order and correlation time profiles were very similar. Correlation times varied from about 8×10^{-11} sec. in the C-3 to C-9 plateau region ($S_{CD} \sim 0.2$) to 3×10^{-11} sec. for the C-15 segment ($S_{CD} \sim 0.04$). It can be concluded therefore that in the hydrophobic interior of the lipid bilayer order and mobility are generally correlated.

Davis (1979) inferred from the fact that his measured spin-spin, (T_2) relaxation values were much less than the T_1 values, that the motions in the lipid bilayer would be better described by two correlation times: τ_1 and τ_2 with $\tau_1 \ll 1/\omega_0 \ll \tau_2$. Brown *et al.* (1983) studied the frequency dependence of both 1H - and ^{13}C -NMR T_1 values and concluded that the following relation applied,

$$\frac{1}{T_1} \cong A\tau_f + BS_{CD}^2 \omega_0^{-1} \quad [6]$$

In this expression the constant A includes contributions from fast segmental motions (e.g. *trans-gauche* isomerizations) characterized by the correlation time τ_f .

The second constant B describes the contribution of slower collective fluctuations of the bilayer to the observed relaxation. These motions were proposed to correspond to relatively long wavelength twist and splay bilayer disturbances of a cooperative nature.

The preceding discussion of deuterium order and relaxation measurements has referred entirely to the liquid-crystalline state of the membrane lipids. When the sample temperature is lowered through the lipid phase transition, a quite broad component corresponding to gel-state lipid begins to appear in the $^2\text{H-NMR}$ spectrum. The gel-state component is superimposed upon the liquid-crystalline quadrupolar doublet indicating that the gel and liquid-crystalline state lipid are in slow exchange on the $^2\text{H-NMR}$ time scale. At approximately 20°C below the phase transition, the liquid-crystalline quadrupolar doublet has all but disappeared (Smith *et al.*, 1979; Davis, 1983). It is not possible to describe this broad gel state component of the $^2\text{H-NMR}$ spectrum in terms of a single order parameter, apparently because in the gel state the rates of rotational diffusion of the lipid molecules are insufficient to satisfy the criteria for axial symmetry on the $^2\text{H-NMR}$ time scale (Davis, 1983). Nevertheless, it is possible to glean information concerning chain motions from gel-state $^2\text{H-NMR}$ spectra through a moment analysis similar to that described earlier for the case of proton NMR. Since specifically deuterated probes are employed, it is possible to evaluate

the variation of the extent of motion with the chain position. For example, Davis (1983) reported that the first moment of the gel state spectra (25°C) of 1,2-[14,14-²H] DPPC was $M_1 = 1.19 \times 10^3 \text{ S}^{-1}$ while for 1,2-[8,8-²H] DPPC, $M_1 = 1.47 \times 10^3 \text{ S}^{-1}$. The smaller value for the C-14 sample indicated that even in the gel phase a head-to-tail gradient in chain flexibility was still present. Even at 0°C this gradient in the value of the first moment was still observed. Although detailed models involving restricted axial diffusion and limited *trans-gauche* isomerization have been proposed (Rice *et al.*, 1981) to interpret the gel state ²H-NMR spectrum and apparently lead to satisfactory spectral stimulations, an evaluation of their correspondence to physical reality awaits the performance of systematic relaxation measurements in order to establish the timescales of the relevant motions (Davis, 1983).

¹⁹F-NMR

Fluorine offers certain distinct advantages over other commonly employed nuclei in the study of membrane hydrocarbon chain mobilities. By virtue of its large magnetogyric ratio, it is much more sensitive in the NMR experiment than either ²H or ¹³C and nearly as sensitive as the proton. Since the natural abundance of fluorine in a membrane is 0%, there are no complications arising from multiple overlapping resonance lines such as those which obscure ¹H-NMR and natural abundance ¹³C-NMR spectra. The synthesis of

specifically fluorine-labelled fatty acids is relatively simple and inexpensive (unlike specific deuteration or ^{13}C enrichment) and permits the location of the nuclear spin probe in the membrane to be predetermined. Both monofluoro- and *geminal* difluoro-labelled fatty acids have been studied and will be considered separately.

Ho and co-workers have been the foremost proponents of the use of *gem*-difluoro fatty acids. Gent *et al.* (1976) measured the spin-lattice relaxation times and Nuclear Overhauser Enhancement (NOE) in preparations of 1-16:0-2-8,8F₂16:0-PC in order to obtain correlation times for specific motions involving the *gem*-difluoromethylene group. It was concluded that the major relaxation mechanism was through dipole-dipole interactions and that chemical shift anisotropy made only a very minor contribution. The calculated correlation time for CF₂ rotation, although of the same order of magnitude as the correlation times for CH₂ rotation obtained from ^1H and ^{13}C data, was significantly larger indicating that the two fluorine atoms might somewhat hinder the motion of that chain segment.

Gent and Ho (1978) were able to analyze the fluorine spectra of various *gem*-difluoromyristic acids intercalated into lamellae of DPPC in terms of chemical shift anisotropy, dipole-dipole considerations and the orientation dependence of these two interactions. The fluorine spectra were demonstrated to be sensitive to temperature and to accurately detect the lipid gel to liquid-crystalline phase transition.

When biosynthetically incorporated into the membrane lipids of *E. coli*, the same dependence on temperature and the lipid phase transition was detected whether in whole cells, isolated membranes or lipid extracts.

Post *et al.*, (1981) developed a pulse sequence which eliminated all broadening due to chemical shift anisotropy and H-F dipole interactions in the fluorine spectrum of 1,2-di-(4,4-F₂,14:0)-PC's. The resulting spectrum was a Pake doublet due entirely to F-F dipole interactions. The separation of the two peaks of the doublet was proportional to S_{FF} and it was demonstrated that values of S_{FF} were in close agreement to values of S_{CD} obtained under similar conditions. Using macroscopically oriented bilayers of PC's containing esterified *gem*-difluoromyristic acids, Engelsberg *et al.* (1982) demonstrated the orientation dependence of the fluorine chemical shift, obtaining a value of 64 ppm for the observed chemical shift anisotropy. By analogy with the experiments of Post *et al.* (1981), upon removal of the broadening due to chemical shift anisotropy through macroscopic orientation of the bilayers, the remaining, predominantly F-F dipole interactions were manifest as a Pake doublet. The separation of the peaks of the doublet was orientation dependent and could be used to calculate a value of S_{FF} which was in good agreement with values of S_{CD} .

Despite this progress in understanding the interactions which contribute to the fluorine spectrum of *gem*-difluoro fatty acids and the extraction of information therefrom,

concern has been expressed regarding the perturbing effects of the *gem*-difluoro group itself. Sturtevant *et al.* (1979) performed differential scanning calorimetric (DSC) measurements on PC's containing a number of positional isomers of *gem*-difluoromyristate. The phase transition temperature relative to DMPC was elevated by 5°C when the difluoro group was located at the 4-position and reduced by 7.4 and 3.8°C when located at the 8- or 12-positions, respectively. The transition enthalpies were always approximately double that of DMPC. Furthermore, the difluoro-containing PC's exhibited markedly nonideal mixing behavior with their nonfluorinated analog DMPC. Longmuir *et al.* (1977) also reported that the transition temperatures of di-(7,7-F₂-18:0)-PC and di-(12,12-F₂-18:0)-PC were reduced by 10 and 6°C, respectively, in comparison to that of DSPC. Oldfield *et al.* (1980) acquired the ¹H-NMR spectrum of 1-myristoyl-2-[2',2',7',7',9',9'-²H₆-8',8'-¹⁹F₂] myristoyl-PC. The quadrupolar splittings of the deuterons adjacent to the *gem*-difluoro group were reduced by approximately 30%, relative to the values obtained at the same position in a nonfluorinated analog. In addition to these physical evaluations of the perturbation associated with the *gem*-difluoro group, the studies of Gent *et al.*, (1978, 1981) indicate that there are biochemical and biological consequences as well. An unsaturated fatty acid auxotroph of *E. coli* was able to incorporate difluoro-containing myristic acids but growth was inhibited when levels of incorporation

exceeded 20% and these fatty acids were unable to support growth for more than two generations. Moreover, there were marked changes in the protein/lipid ratios and transport properties of these membranes.

Monofluoro-substitution has proven to be much less perturbing than difluoro-substitution while retaining all of the potential advantages inherent in the fluorine nucleus. Birdsall *et al.* (1971) first characterized the linewidths of monofluorostearic acids intercalated into egg PC vesicles as a function of ^{19}F substituent position and temperature. The linewidths were observed to increase with decreasing temperature and to decrease steeply toward the terminal methyl group of the alkyl group. These changes could be attributed to the molecular motion of the chain in the bilayer although no quantitative analysis was attempted. Esfahani *et al.* (1981) incorporated 16-monofluoropalmitic acid into yeast cell membranes and observed that qualitatively the fluorine spectral linewidth decreased upon isolating the membrane lipids.

A systematic evaluation of the acceptability of monofluoropalmitic acids as membrane probes was undertaken by McDonough *et al.* (1983) by applying physical, biochemical and biological criteria. DSC studies of various isomeric monofluoropalmitic acids synthetically incorporated into PC's demonstrated that a monofluoro-substituent near the carbonyl group of palmitic acid had only a modest effect on the thermotropic behavior of the PC's and that substitutions

in the center or towards the methyl terminus had very little effect on the T_m relative to DPPC. Furthermore these monofluoro-substituted PC's exhibited nearly ideal mixing in all proportions with DPPC. The monofluoropalmitic acids (MFPA) could replace up to 80% of the membrane glycerolipid fatty acids of *A. laidlawii* B without affecting growth or survival of this organism. The presence of these compounds did not alter the polar headgroup composition or lipid/protein ratio of the *A. laidlawii* B membrane. Moreover, all MFPA's tested were biosynthetically incorporated as well as palmitic acid itself and distributed equitably between the various membrane glyco- and phospholipids. It was concluded that mono-fluorinated palmitic acids are considerably less perturbing than *geminal*-difluorinated fatty acids.

Macdonald *et al.* (1983) presented a quantitative description of the ^{19}F -NMR spectrum of MFPA's incorporated into membrane lipids of *A. laidlawii* B which permitted the determination of an orientational order parameter, S_{m01} . It was demonstrated that the order profile obtained via ^{19}F -NMR was qualitatively similar to that obtained via ^1H -NMR techniques. When small quantities of MFPA's were incorporated in the presence of a large excess of structurally diverse fatty acids, it was demonstrated that the order profile and temperature dependence of the order parameters were sensitive to the presence of these various chain structural substituents. For example, methyl-branch substitution

induced a local ordering while *trans*-unsaturation induced a local disordering effect. When compared at 37°C the order parameters from fatty acyl species to species varied inversely with increasing temperature above the particular phase transition. When normalized with respect to the phase transition temperatures, it was observed that those fatty acyl structures which were methyl-branched or *trans*-unsaturated were generally more ordered than, for example, straight-chain saturated fatty acids. The utility of ¹⁹F-NMR of monofluorinated fatty acids as a nonperturbing, sensitive and versatile technique was convincingly demonstrated.

If any one general conclusion can be drawn from this review of NMR techniques as applied to membrane fatty acyl chains, it is that no one nucleus and no single NMR methodology provides all the answers. Rather the information provided by various techniques complements and enhances that obtained from another. Analysis of ³¹P spectra of phospholipid dispersions can furnish insights into the overall motions of the lipid molecules and of the headgroup region in particular, but more specific information requires that other nuclei be observed. In principle, an order parameter can be extracted from analysis of ¹H, ¹³C and ¹⁹F NMR spectra but the overlap of methylene resonances in ¹H and natural abundance ¹³C spectra permit only an average order parameter to be obtained (Hentschel *et al.*, 1978). The same proviso applies to the method of moments in ¹H-NMR and to proton relaxation measurements which are further complicated

by possible spin-spin diffusion effects (Petersen and Chan, 1977). The synthesis of specifically deuterated or ^{13}C -enriched lipids is time consuming and expensive but provides relatively unambiguous and detailed order and mobility information from spectral analysis and relaxation measurements. Even so, precise data is restricted to the liquid-crystalline phase. ^{19}F -NMR can provide information on both order and mobility at precise locations but concerns arise regarding the possible perturbing effects of the *geminal*-difluoro group. Even when these concerns are alleviated by the use of monofluorinated fatty acids, the spectral analysis is complicated by the necessity of considering both chemical shift anisotropy and dipolar interactions.

It is clear that normal cell function requires fluid membrane bilayers. Altering membrane "fluidity" has numerous physiological consequences in the absence of any compensating regulatory mechanism. Studies of the relationship between membrane lipid fluidity and phase state and cell growth have established that a minimum level of membrane lipid fluidity is required for normal cell growth, that gel state lipid domains do not support at least some essential membrane functions and, that there exists some upper limit on membrane lipid fluidity compatible with cell growth (for a recent review, see McElhaney, 1984). Not only cell growth but also passive permeability and active transport across the membrane, as well as the activities of membrane bound enzymes, are highly influenced by the phase

state and fluidity of the membrane lipids (for a review, see McElhaney, 1982a).

Most organisms have developed mechanisms which enable them to respond to environmental changes in such a fashion as to maintain some constant level of membrane lipid fluidity and these are collectively referred to as "homeoviscous" or "homeophasic" adaption mechanisms. These strategies most often involve alterations in the fatty acyl constituents of membrane lipids because this is the most effective means through which to modulate membrane lipid fluidity. While the relationship between altered fatty acid structure and changes in the temperature of the lipid phase transition has been firmly established using techniques such as differential scanning calorimetry (DSC) (e.g., McElhaney, 1982b), these studies provide few insights into the molecular details of conformation and dynamics which result in the observed fluidity changes. An appreciation of these molecular details is a prerequisite to resolving many of the fundamental issues confronting the membranologist. How do various fatty acyl structural substituents contrive to modulate the temperature of the lipid phase transition? Is there a common feature which relates various specific structural substituents to the phase transition temperature? Is it possible to define some point at which membrane lipids become "hyperfluid" and incapable of functioning as biologically effective membrane constituents? These and other questions must be addressed by the application of

techniques sensitive to structural and dynamical parameters.

The primary goal of the research to be described in this thesis has been to explore the relationship between fatty acid structure and the physical characteristics of membrane lipids. ^{19}F -NMR of monofluoropalmitic acids was chosen as the preferred spectroscopic technique because NMR techniques in general are capable of providing both structural-conformational and dynamic information, the synthesis of specifically monofluorinated fatty acids is relatively simple and inexpensive, and because these probes have been proven to be relatively non-perturbing, sensitive and versatile monitors of membrane lipid order, capable of discerning subtle effects arising from differences in fatty acid structure and physical state (McDonough, *et al.*, 1983; Macdonald *et al.*, 1983). In the interests of biological relevance and because of the ease with which a membranous venue is obtained, these studies have utilized membranes of *A. laidlawii* B in which to investigate the effects of fatty acyl structural variants. This organism has no cell wall and contains only a single membranous structure, the plasma membrane, so that the isolation of relatively homogeneous membrane preparations is facile (Razin, 1975). *A. laidlawii* B will readily incorporate a variety of exogenously supplied fatty acids (McElhaney, 1974) and, when *de novo* fatty acid biosynthesis is inhibited with, for example, the compound avidin, it is possible to produce membranes which are virtually homogeneous with respect to a particular fatty

acid (Silvius and McElhaney, 1978a). This property permits the fatty acid composition and the physical state of the membrane lipids to be controlled and varied over a wide range almost at will. "Homogeneous" membranes of *A. laticauda* B may be considered in a sense as a bridge between, on the one hand, the artificiality of pure lipid/water systems and, on the other hand, the complex heterogeneity of conventional biological membranes.

The studies to be described here may be divided into two categories. In the first section a mathematical description of the ^{19}F -NMR line shape is presented which permits the extraction of an order parameter, S_{m01} . Included here are evaluations of the fidelity of the spectral simulations employed, comparisons of the fluorine order parameters with those obtained using other techniques such as ^1H -NMR, and results which suggest that ^{19}F -NMR may permit the characterization of gel state lipids in terms of a useful order parameter formalism. In the next category are those studies dealing with the effects of specific structural substituents in the fatty acyl chains upon the fluorine spectrum of monofluoropalmitic acids. Representatives of each class of naturally occurring fatty acid have been investigated including straight-chain saturated, unsaturated, methyl-branched, and alicyclic ring-substituted species. In combination with an independent evaluation of the physical state of the membrane lipids by DSC, these ^{19}F -NMR studies have permitted the formulation of

an unified picture of the manner in which fatty acyl structure can modulate the physical properties of membrane lipids.

II. MATERIALS AND METHODS

Materials

Biochemicals

The straight-chain saturated and *trans*-unsaturated fatty acids used in these studies were purchased from Nu-Chek Prep Co. (Elysian, Minn.). The isomeric series of *cis*-octadecenoic acids were a kind gift of Dr. F.D. Gunstone, University of St. Andrews, Scotland, U.K. The cyclopropane and methyl-branched chain fatty acids were products of Analabs (North Haven, Conn.). Avidin was obtained from Sigma (St. Louis, Mo.) while bovine serum albumin (fraction V, fatty acid poor) was obtained from Miles Biochemicals (Elkhart, Ind.) and further delipidated as described by Chen (1967). Bacto-peptone, bacto-heart infusion broth and bacto-yeast extract were produced by Difco (Detroit, Mich.). Penicillin G was purchased from Ayerst Laboratories (Montreal, Quebec).

General Chemicals

The precursors used in the synthesis of the various isomeric monofluoropalmitic acids were purchased from either Aldrich Chemicals (Milwaukee, Wis.), Fisher Scientific (Edmonton, Alta.) or K and K (Irvine, CA). Tetrabutylammonium iodide and silver fluoride, which were employed as precursors in the synthesis of tetrabutylammonium fluoride, were products of Aldrich Chemicals and ICN Pharmaceuticals

(Plainview, N.Y.), respectively. The various inorganic salts, catalysts and organic solvents used were obtained from either Fisher Scientific, Aldrich Chemicals or Baker Chemicals (Phillipsburg, N.S.) and were of reagent grade or better. All solvents were routinely redistilled prior to use.

Chromatography

Bio-Sil A (200-400 mesh) was purchased from Bio-Rad (Mississauga, Ont.) while silica gels G and H were obtained from E. Merck (Darmstadt, G.F.R.). Both Bio-Sil A and the silica gels were washed with chloroform and methanol and dried prior to use. The gas chromatographic columns (10% diethylene glycol succinate on Anakron) were purchased prepacked from Analabs. The gases used (ultra-high purity hydrogen, 'zero-gas' grade air, and purified grade helium) were obtained from Union Carbide Canada Ltd.

Methods

Growth of *Acholeplasma laidlawii* B

Acholeplasma laidlawii B (originally obtained from Dr. G. Edwards, Wellcome Research Laboratories, Beckenham, U.K.) was cultured in a delipidated growth medium which was prepared as described by Silvius and McElhañey (1978a). This growth media was supplemented with 0.4% (w/v) bovine serum albumin, 0.25% (w/v) glucose and 10^5 I.U./litre of penicillen G prior to inoculation. Fatty acids were added

as solutions in a small quantity of ethanol (~0.3% of the culture volume). Where applicable, avidin was added to a level of 3 mg/litre. Cultures were grown statically at 37°C, with the exception of those supplemented with 18:1cΔ8, 9, 10 or 11 and 19:0cp,cΔ9, which were grown at 32°C. Cell growth was monitored turbidometrically at 450 nm (Maniloff, 1969) and the cultures were harvested in late log phase by centrifugation at 13,000 x g for 15 min at 4°C.

Membrane Isolation

Harvested cells were resuspended in one-fifth culture volume of 'β-buffer' (0.154 M NaCl, 0.05 M Tris-HCl, 20 mM β-mercaptoethanol, pH 7.4), recentrifuged, and the plasma membranes were then isolated basically according to the procedure of Pollack *et al.* (1965). The cell pellet was resuspended in one-fifth culture volume of distilled water and incubated at 37°C for 30 min. This suspension was then centrifuged at 8,000 x g for 10 min to remove debris and unlysed cells, the resulting supernatant was centrifuged at 30,000 x g for 30 min to isolate the membrane fraction, and the pellet was washed once by recentrifugation from β-buffer diluted twenty-fold with 95% deuterium oxide.

Nuclear Magnetic Resonance

The membrane fraction isolated as described above was resuspended in a minimal volume (~1.5 ml) of β-buffer diluted twenty-fold with 95% deuterium oxide. ¹⁹F-NMR

spectra were collected at 254.025 MHz on a Bruker HXS-270 spectrometer, operating in the Fourier transform mode and using quadrature detection, at a spectral width of $\pm 50,000$ Hz. Bessel filters with a filter width of $\pm 100,000$ Hz were employed. The probe was maintained at the specified temperature to within $\pm 1^\circ\text{C}$. Temperature variation was achieved by passing an air-stream over the cooling coil of a cryocool unit and subsequently over the probe and sample. For very low temperature experiments the air flow was directed additionally through a stainless steel coil immersed in liquid nitrogen. An electric heating coil was used to balance the cooling of the airstream and achieve temperature control. Samples were equilibrated for 30 min at a particular temperature prior to data acquisition.

A one pulse experiment was routinely employed to obtain ^{19}F spectra and may be described as follows:

$$[\text{P2-A-D2-D5}]_N \quad [1]$$

P2 is the pulse length (corresponding to 15 μsec or an approximately 75° flip angle), A is the delay between the pulse and data acquisition (equalling 10 μsec or one dwell time), D2 is the acquisition time (set by the spectral width and corresponding to 20 msec in our case), D5 is the post acquisition delay which permits the fluorine nuclei to return to equilibrium prior to the next pulse (200 msec) and N corresponds to the number of repetitions. Typically 20 K

acquisitions were averaged for samples in which the acquisition temperature was above the lipid phase transition and 100 K acquisitions were averaged at lower temperatures. The distortion of the first three points of the free induction decay (FID), which is associated with the receiver dead time, was corrected by a smooth extrapolation of the FID back to time zero such that the signal intensity of the early portion of the FID closely approximated a t^2 time dependency in the in-phase channel and a t time dependency in the out-of-phase channel (Bloom *et al.*, 1978). The signal-to-noise ratio was enhanced with an exponential multiplication which corresponded to a line broadening of either 50 Hz or 100 Hz and the FID was Fourier transformed to 2 K data points in the real domain. Provided that the FID had been properly corrected for the receiver dead-time distortion, the final spectrum required only zero-order phase corrections and displayed no baseline distortion.

Longitudinal relaxation times (T_1) were measured using an inversion-recovery pulse sequence (Levy and Peat, 1975) which can be written:

$$D5-[P1-D1-P2-A-D2-D5]_N \quad [2]$$

where P1 is the 180° pulse length (40 μ sec), D1 is the variable delay between the inversion and the sampling pulses (at least 8 to 10 different values of D1 were used per T_1 measurement), P2 is the 90° pulse length (20 μ sec), A is the

delay between the end of the sampling pulse and the beginning of data acquisition (10 μ sec), D2 is the acquisition time (20 msec), D5 is the post acquisition delay (800 msec) and N is the number of repetitions of the pulse sequence (typically 10,000 at 310°K). The final spectra were obtained as described above for the one-pulse experiment. The T_1 values were obtained by determining the slope in a plot of $\ln(S_0 - S_t)$ versus t where S_0 is the thermal equilibrium magnetization.

Lipid Extraction

After analysis by ^{19}F -NMR, the membrane lipids were extracted using a modification of the method of Bligh and Dyer (1959). The sample of interest was suspended in 8 ml of ' β -buffer', 20 ml of methanol was added and the mixture was warmed to -60°C for 10 min to denature the protein. Chloroform (30 ml), water (20 ml) and chloroform (40 ml) were then added sequentially, shaking the mixture thoroughly after each addition. The phases were separated by centrifugation, the upper (aqueous methanol) phase and the interfacial material were removed by aspiration, and the lower (chloroform) phase was evaporated to dryness. This total lipid extract was applied in a small volume of chloroform to a column of Bio-Sil A (5-10 gms) packed in chloroform. The column was eluted with 5 column volumes of chloroform to yield the neutral lipids and then with 5 column volumes of methanol to yield the polar lipids. The polar lipid fraction

was evaporated to dryness, dissolved in a minimal volume of benzene, quick frozen, and placed under high vacuum overnight yielding a dry powdery sample of polar lipids.

Differential Scanning Calorimetry of Lipid Samples

The dry polar lipid samples obtained as described above were resuspended in either water or ethylene glycol/water, 1:1 (v/v) by a combination of heating to 50°-60°C and mild vortexing. DSC endotherms were obtained using a Perkin-Elmer DSC-2 equipped with a thermal analysis data station (TADS) and subambient temperature cooling unit. Generally a heating scan rate of 5°C/min was employed. T_m , ΔT_{10-90} and transition enthalpies (when determined) were obtained using the partial areas program which accompanied the TADS unit.

Fatty Acid Analysis

The fatty acid composition of the polar lipid fraction was determined by gas chromatography as described by Silvius and McElhaney (1978b). Lipids were dissolved in anhydrous methanol (up to 10 mg/ml) containing 3% sulphuric acid and reacted for 2 hr at 70°C. The cooled reaction mixture was mixed with 2 volumes of water and extracted twice with one volume of hexane which was dried (Na_2SO_4) and evaporated under nitrogen gas. The resulting fatty acid methyl esters were analyzed by gas chromatography on columns of 10% diethylene glycol succinate on Anakron in a Hewlett-Packard 5700 A gas chromatograph. The signal from the flame

ionization detector (which is proportional to the mass of the organic compounds in the column effluent) was coupled to a Hewlett-Packard 3370B integrator for analysis. The fatty acid methyl esters were identified by their retention times in comparison with those of standards of known composition.

Synthesis of Fluorinated Fatty Acids

Mono-fluorinated fatty acids of the general formula $\text{CH}_3(\text{CH}_2)_m\text{CHF}(\text{CH}_2)_n\text{COOH}$, where $m + n = 13$, were synthesized from the corresponding keto acyl methyl esters following a procedure modified from that of Birdsall *et al.* (1971). The keto acyl methyl esters were built up from the appropriate precursors using a Grignard condensation as described by Hubbell and McConnell (1971). The overall reaction pathway is outlined in Figure 1.

Preparation of VI

The commercially available dicarboxylic acids I(n), where $n = 6, 8, 10$ or 12 , were first converted to the corresponding di-methyl esters, II(n), and subsequently to the corresponding acid ester, III(n), by the methods of Jones (1947) and purified by vacuum distillation. The acid ester, III (4), was obtained commercially. The acid esters were converted in good yields to acid chlorides, IV (n), using thionyl chloride as described by Morgan and Walton (1935), the excess solvent and thionyl chloride were removed by distillation, and the acid chlorides were used

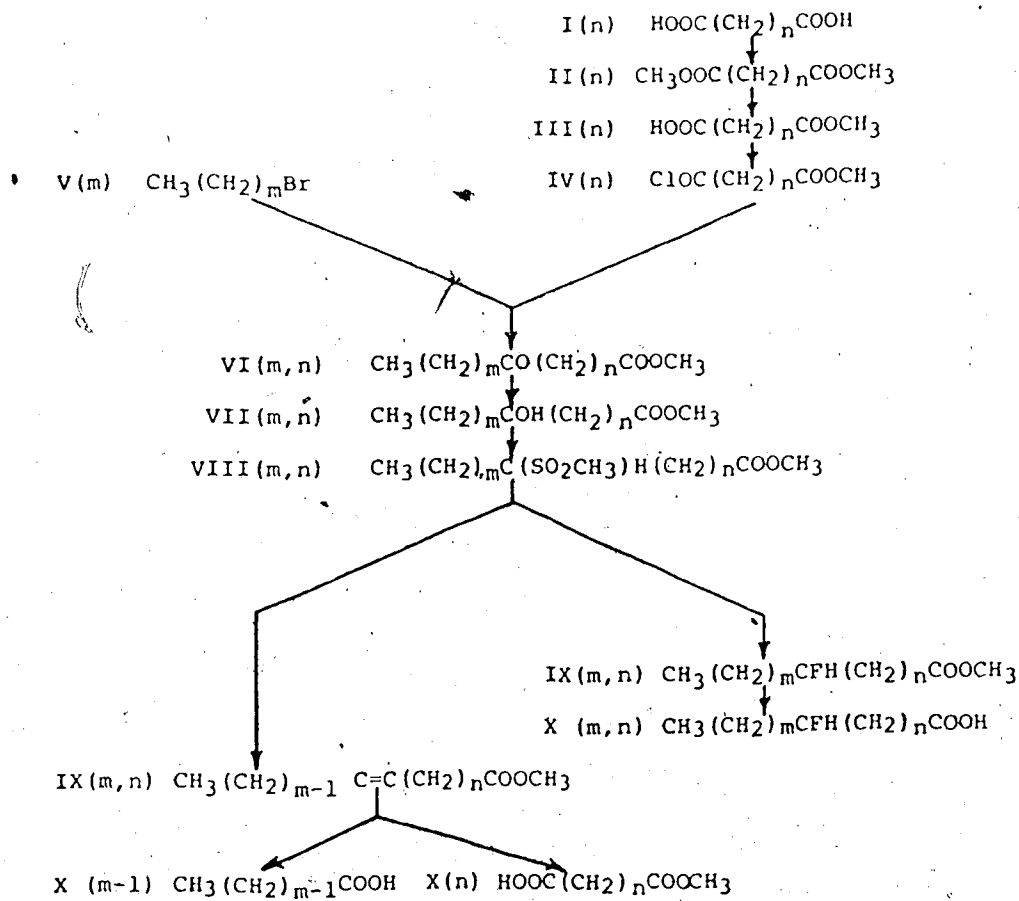


Figure 1: Chemical synthesis of the monofluoropalmitic acids.

immediately without further purification.

The entire series of alkyl bromides V (m), where m = 1, 3, 5, 7 or 9, were acquired commercially and condensed in a Grignard reaction with the appropriate acid chloride IV (n) exactly as described by Hubbell and McConnell (1971) to yield the keto acyl methyl ester VI (m,n). The yield from this reaction averaged 40%. The keto acyl methyl ester products were purified by two recrystallization from hexane followed by silicic acid column chromatography.

Preparation of X (m,n)

The keto acyl methyl esters VI (m,n) were converted to monofluorinated fatty acids X (m,n) using the reaction scheme outlined by Birdsall *et al.* (1971). The ketone function was first reduced to a hydroxy function using NaBH₄ (yield ~90%) and the reaction product, VII (m,n), was purified by silicic acid column chromatography. The hydroxy derivative was then mesylated using methane sulfonyl chloride in the presence of triethylamine, and the mesylated fatty acyl methyl ester XIII (m,n) was extracted into methylene chloride, concentrated *in vacuo* and used without further purification (yield ~98%). This mesyl derivative was dissolved in dry acetonitrile in the presence of five equivalents of dry tetrabutylammonium fluoride and reacted for three days at room temperature to yield 30-40% of a monofluoropalmitic acyl methyl ester, IX (m,n). An unsaturated fatty acyl methyl ester IX (m-1,n) was a major

byproduct of the fluorine substitution reaction and was removed by oxidation to a short chain fatty acid, X (m-1), and an acid ester, X (n) using the Von Rudloff procedure (Christie, 1973). The monofluoropalmitoyl methyl ester was readily separable from the products of the Von Rudloff oxidation by a combination of acid-base extraction, crystallization from acetone and then hexane, followed by silicic acid column chromatography. The methyl ester was converted to the free monofluoropalmitic acid X (m,n) by heating in methanolic KOH (Silvius and McElhaney, 1978b) and was then crystallized from 70% ethanol in water. The purity was estimated to be greater than 99.9% as judged from the results of analytical thin-layer and gas-chromatographic analysis.

III. THEORY

General

The dominant interactions in the NMR experiment for a spin 1/2 nucleus such as fluorine or phosphorus are the Zeeman coupling to the external magnetic field and dipole-dipole interactions between spins (Wennerstrom and Lindblom, 1977). The spin Hamiltonian may thus be written:

$$H = H_z + H_D \quad [1]$$

Since the Hamiltonian is orientation dependent, in the presence of molecular reorientations it will vary with time. The time-independent part, H_0 , determines transition frequencies and intensities while the time-dependent part, $H_1(t)$, determines relaxation and line-broadening effects. Formally one can write:

$$H = H_0 + H_1(t) \quad [2]$$

$$H_0 = \bar{H}_z + \bar{H}_D \quad \text{and} \quad H_1(t) = (H_z - \bar{H}_z) + (H_D - \bar{H}_D) \quad [3]$$

The bar denotes an average over a time that is long compared to the inverse of the coupling (in Hertz) that is modulated by the motion. $H_1(t)$ is defined so that it has zero time

average.

Chemical Shift Anisotropy

The Zeeman coupling is

$$H_z = I \cdot \sigma \cdot H \quad [4]$$

and is influenced by the chemical shift tensor, σ , which contains both orientation-dependent and orientation-independent terms. The following discussion is based primarily on the work of Niederberger and Seelig (1976). The components of the static chemical shift tensor of the fluorine nucleus in a monofluoropalmitic acid will be denoted with σ'_{kk} ($k = 1, 2, 3$). These components are defined as follows: σ'_{33} lies along the C-F bond direction, σ'_{22} is perpendicular to σ'_{33} in the H-C-F plane, and σ'_{11} is parallel to the normal to that plane coincident with the long axis of the fatty acyl chain (see Figure 2). In solution, due to the rapid reorientations of the molecule, only the average value σ'_i (corresponding to the resonance frequency ν_i) is observed

$$\sigma'_i = 1/3(\sigma'_{11} + \sigma'_{22} + \sigma'_{33}) \quad [5]$$

The motion of the fatty acyl chain in a lipid bilayer is, anisotropic and may best be described as a rapid rotation about the molecular long axis perpendicular to

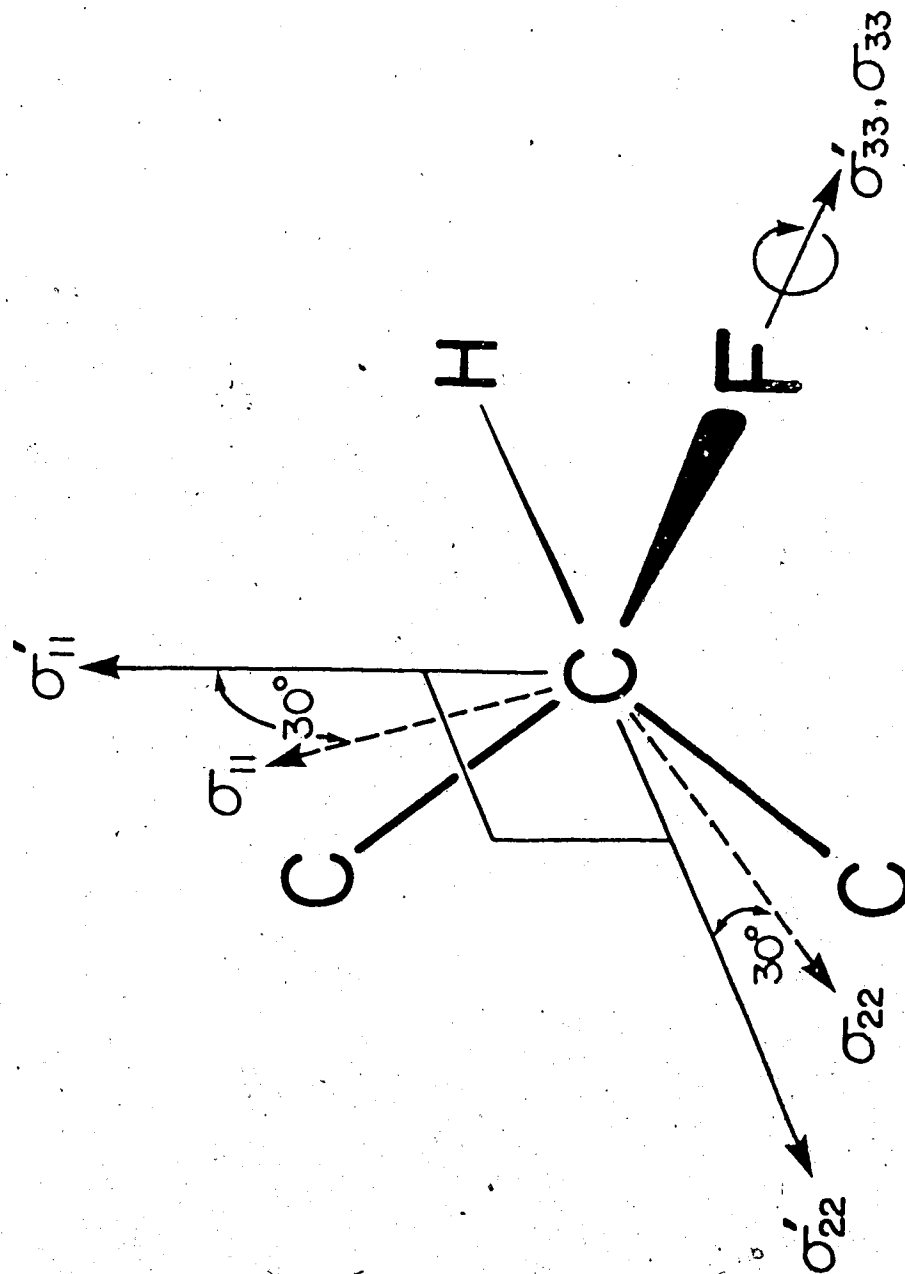


Figure 2: Geometry of the H-C-F group and the coordinate system of the fluorine chemical shift tensor.

the plane of the bilayer (i.e., axial symmetry). The observable chemical shift is then given by:

$$\sigma = \sigma'_i + \frac{2}{3} \sigma'_a \left(\frac{3\cos^2\theta - 1}{2} \right) \quad [6]$$

with:

$$\sigma'_a = (\sigma'_{11}S_{11} + \sigma'_{22}S_{22} + \sigma'_{33}S_{33}) \quad [7]$$

The term θ represents the angle between the magnetic field and the axis of motional averaging (i.e., the normal to the bilayer), while S_{kk} denotes the order parameter of the k th axis (Saupe, 1964). Only two order parameters are independent since

$$\sum_{k=1}^3 S_{kk} = 0 \quad [8]$$

The motional averaging in a lipid bilayer (cylindrical or axial symmetry) confers a pseudo-axial symmetry upon the chemical shift tensor. Therefore one can state:

$$\sigma_{||} = \sigma'_{11} \quad [9]$$

and:

$$\sigma_{\perp} = 1/2(\sigma'_{22} + \sigma'_{33}) \quad [10]$$

The presence of this axis of symmetry dictates that $S_{22} = S_{33}$ and only a single order parameter remains independent and is therefore sufficient to define the order of the entire coordinate system. Consequently, the observable chemical shifts can be written:

$$\sigma'_{\perp} = 1/3(2\sigma_{\perp} + \sigma_{\parallel}) \quad [11]$$

and:

$$\sigma'_a = S_{11}(\sigma_{\parallel} - \sigma_{\perp}) \quad [12]$$

The resonance frequency is then:

$$\nu = \nu_i - \nu_a \left(\frac{3\cos^2\theta - 1}{2} \right) \quad [13]$$

where:

$$\nu_a = 2/3\sigma'_a \nu_0 \quad [14]$$

with ν_0 equalling the instrumental fluorine frequency. Equation 13 defines the resonance position for any one orientation of the planar bilayer with respect to the external magnetic field, H_0 . If the bilayers are distributed at random, as in a suspension of membrane fragments, all orientations are possible and the shape of the resulting "powder-type" spectrum is the sum of the resonances of all

possible orientations, θ .

It is possible to define a probability function, $p(\nu)$, so that $p(\nu)d\nu$ describes the fraction of spins with resonance frequencies between ν and $\nu + d\nu$. $p(\nu)$ is found to be (Abragam, 1961)

$$p(\nu) \propto \left\{ 1 - \frac{2(\nu - \nu_i)}{\nu_a} \right\}^{-\frac{1}{2}} \quad [15]$$

This probability function defines the overall shape of the "powder-type" spectrum in the absence of dipolar line-broadening interactions. For example, when $\theta = 0^\circ$, $p(\nu) \propto 1/3$, when $\theta = 54.44^\circ$, $p(\nu) \propto 1$, and when $\theta = 90^\circ$, $p(\nu) \propto \infty$.

Dipolar Interactions

The H-F dipolar coupling is:

$$H_D = \gamma_H \gamma_F h^2 \sum_{i,j} \frac{1}{r_{ij}^3} (3\cos^2\psi_{ij} - 1) (\hat{I}_i \cdot \hat{I}_j - 3I_{iz}I_{jz}) \quad [16]$$

where the summation is performed over all F-H pairs, r_{ij} is the F-H internuclear distance and ψ is the angle between the external magnetic field and the F-H internuclear vector (Griffin, 1981). It is convenient to separate the dipolar Hamiltonian into time-dependent and time-independent terms (Bloom *et al.*, 1975).

$$H_D = \bar{H}_D + [H_D(t) - \bar{H}_D] \quad [17]$$

For cases in which $\bar{H}_D \neq 0$, the experimental line shape will be governed by \bar{H}_D , which contains both orientation-dependent and orientation-independent terms:

$$\bar{H}_D = \bar{H}_{Di} + \bar{H}_{Da} \quad [18]$$

In the liquid-crystalline state rapid diffusion of the lipid molecules effectively reduces intermolecular H-F dipolar interactions to a minimum. The residual dipolar interactions are due completely to the interactions among H-F pairs on individual lipids. In the presence of rapid long-axis rotational reorientations of the lipid molecules the individual H-F dipolar interactions are projected onto the axis of motional averaging so that the orientation-dependent term is expressed:

$$\bar{H}_{Da} = \bar{H}_D \cdot \frac{1}{2} (3\cos^2\theta - 1) \quad [19]$$

where θ is again the angle between the axis of motional averaging (the lipid long axis) and the external magnetic field. \bar{H}_{Di} represents the value of the projection of the H-F dipole interaction onto the axis of motional averaging. The total H-F dipolar interaction may then be written:

$$\bar{H}_D = \bar{H}_{Di} + \left(\frac{3\cos^2\theta - 1}{2} \right) \sum_{ij} \bar{H}_{Dlij} \quad [20]$$

where θ is as defined previously and the summation is over

all intramolecular H-F pairs. This equation describes the manner in which the fluorine resonance linewidths will vary as a function of the orientation of the planar bilayer with respect to the magnetic field. In particular, the resulting linewidths will be minimal at the magic angle of 54.44° (Bloom *et al.*, 1975; McLaughlin *et al.*, 1975). Defining the Gaussian linewidth at half-height as:

$$(\delta\nu)_{1/2} = 2.36 \Delta \quad [21]$$

Δ can be decomposed into constant and angular-dependent portions (Niederberger and Seelig, 1976) and

$$\Delta = \Delta_0 + \Delta_1 \left(\frac{3\cos^2 \theta - 1}{2} \right) \quad [22]$$

Assuming that the intrachain dipolar interactions are modulated by the same molecular motions as the chemical shift anisotropy (Gent and Ho, 1978), then one can write:

$$\Delta = \Delta_0 + \Delta_1 \left(\frac{3\cos^2 \theta - 1}{2} \right) S_{11} \quad [23]$$

where S_{11} represents the order parameter of the lipid long axis. The orientation-dependent term, Δ_1 , represents the contribution of intramolecular dipole-dipole interactions, and the constant term, Δ_0 , is the residual linewidth after suppression of Δ_1 (i.e., by decoupling) and arises predominantly from intermolecular dipole-dipole interactions.

¹⁹F-NMR Line Shape

The ¹⁹F-NMR line shape of a sample of randomly distributed planar bilayers is considered to consist of individual resonances centered at frequency, ν^* , corresponding to particular orientations, θ , of the H-C-F segments with respect to the magnetic field. The observed frequency, ν , varies as a function of orientation as described by equation 13. The Gaussian linewidth at a particular orientation varies according to equation 22. The probability of observing a resonance line at frequency ν is described by equation 15. The intensity of an individual resonance line centered at frequency ν^* as a function of the observed frequency ν can be described by a normalized Gaussian of the form (Niederberger and Seelig, 1976):

$$I(\nu - \nu^*) = \frac{1}{\sqrt{2\pi}} \cdot \frac{1}{\Delta} \text{EXP} \left\{ -\frac{(\nu - \nu^*)^2}{2\Delta^2} \right\} \quad [24]$$

The total absorption intensity, $S(\nu)$, at a frequency, ν , is then the sum over all overlapping resonances, ν^* , weighted with their corresponding probabilities $p(\nu^*)$

$$S(\nu) = \int_{-\infty}^{+\infty} I(\nu - \nu^*) p(\nu^*) d\nu^* \quad [25]$$

The observed ¹⁹F-NMR line shape is then $S(\nu)$ as a function of different individual frequencies, ν . Essentially the shape of the spectrum is governed by the chemical shift anisotropy, by the linewidth parameters Δ_0 and Δ_1 , and by

the order parameter S_{11} .

Order Parameters

Flexible molecules which are restricted in their motions are said to exhibit order. The angular distribution of fluctuations of the CH_2 monomers in a polymethylene chain is conventionally described by the second rank, symmetric, traceless tensor S (Saupe, 1964) called the order parameter tensor (or ordering matrix). The matrix components are a measure of the angular fluctuations of the axes (x, y, z) of a molecule-fixed coordinate system with respect to some arbitrarily defined axis and are given by:

$$S_{ij} = \langle (3\cos\theta_i\cos\theta_j - \delta_{ij})/2 \rangle \quad [26]$$

where the brackets denote a time average. The subscripts i and j refer to the molecule-fixed axes (x, y, z) while θ_i and θ_j refer to the angle between these axes and the arbitrarily defined reference axis. For a molecule-fixed axis system exhibiting no particular symmetry in its motion, at least five independent components of the matrix must be identified to define the order parameter tensor (Saupe, 1964). Since the existence of motional planes of symmetry reduces the number of independent components required to completely specify the order parameter tensor, the chosen axis system should exhibit the highest possible degree of symmetry in its motion. A single plane of symmetry reduces the number of

independent matrix components to three. In theory there will always exist some principal axis system containing at least two orthogonal planes of symmetry which is capable of reducing a symmetric tensor of second rank to its diagonal form. Proper identification of this principal axis system reduces the number of independent tensor elements to two. If an axis system with 3-fold or higher symmetry exists (axial symmetry), only one independent element is required to specify the complete order parameter tensor (Saupe, 1964).

It has been routinely assumed in the literature that rotation of the lipid molecules about an axis normal to the plane of the lipid bilayer is sufficiently fast to meet the condition of axial symmetry, at least in the liquid-crystalline state (Seelig, 1977; Seelig and Seelig, 1980; Jacobs and Oldfield, 1981). While this assumption may represent a less than perfect approximation (Meraldi, 1981; Higgs & MacKay, 1977; Post *et al.*, 1981), it drastically simplifies the analysis of the anisotropic motions of the methylene segments. As described earlier, axially symmetric rotation about the normal to the bilayer was invoked to describe the orientation dependence of the chemically shifted fluorine resonance lines and their dipolar broadening. Thus the angular fluctuations of the methylene segments about the axis of motional averaging may be quantitated in the ^{19}F -NMR spectrum via a single order parameter, S_{11} , provided that the assumption of effective axial symmetry is valid.

In order to render the physical meaning of the value of the order parameter more clear, it is convenient to introduce the concept of S_{m01} . The following discussion is based upon the studies of Seelig and Seelig (1980) and Petersen and Chap (1977). An arbitrary axis called the director, \vec{d} , is first defined to lie parallel to the normal to the plane of the bilayer. It is about this director that the Hamiltonian is assumed to be axially symmetric. S_{m01} quantitates the angular excursions of the methylene segments about the director. An acyl chain in the all-*trans* configuration, aligned parallel to the bilayer normal and undergoing rotations about its long molecular axis, would have a value of $S_{m01} = 1$ for each methylene segment. At the other extreme of completely statistical movement through all angles of space, S_{m01} equals zero.

In general NMR techniques measure some order parameter S which relates to the angle β between the director \vec{d} and the particular molecular interaction vector \vec{r} (see Figure 3) such that:

$$S_{\beta} = \overline{1/2(3\cos^2\beta - 1)} \quad [27]$$

where the bar represents the time average. Different NMR techniques pertain to different molecular interaction vectors. For example, ^2H -NMR of deuterated lipids measures the anisotropy of the motion of the C-D bond, ^1H -NMR is sensitive to the orientation of the *geminal* interproton

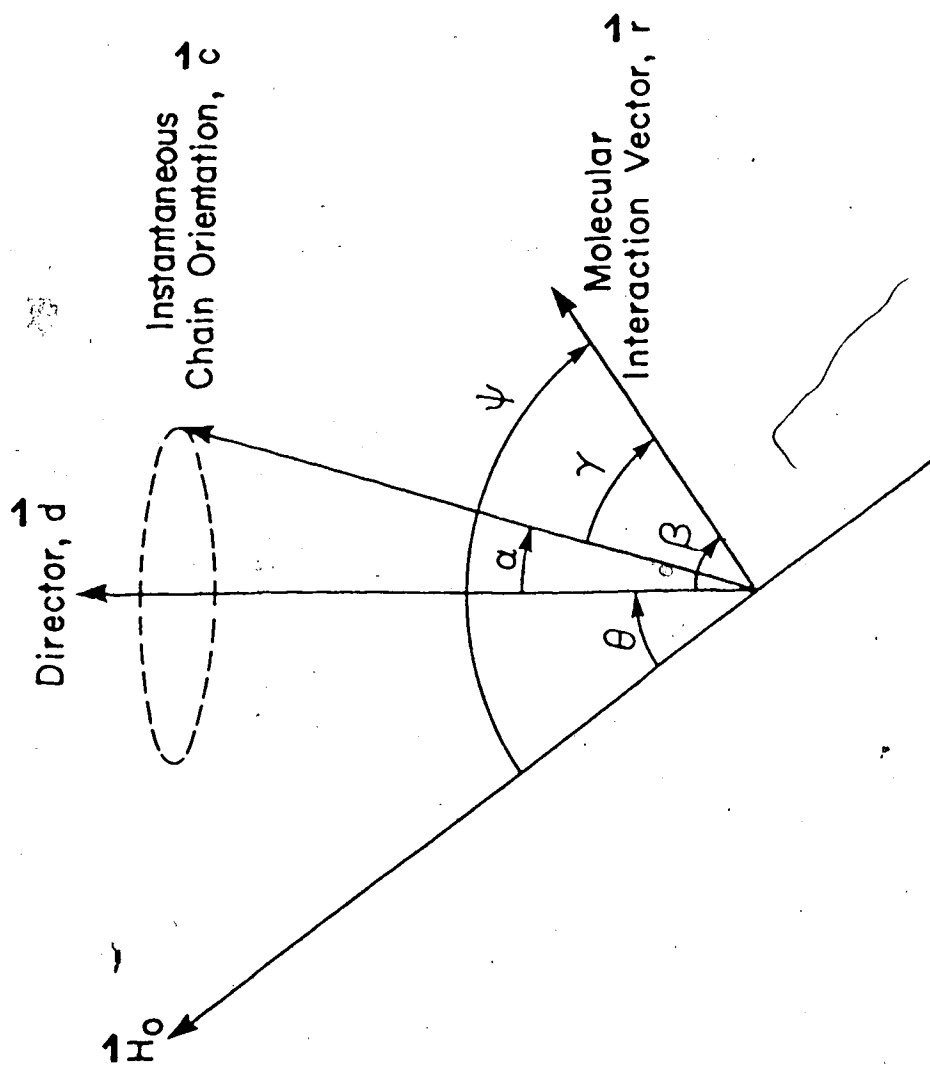


Figure 3: Illustration of the vectors and angles relevant to lipid chain motion. \vec{H}_0 is the direction of the applied magnetic field. The director, \vec{d} , is normal to the bilayer surface. Adapted from Petersen and Chán (1977).

vector. ^{19}F -NMR spectra of *gem*-difluoro substituted fatty acids when depaked can quantitate the orientation of the *geminal* interfluorine vector, while ^{19}F -NMR spectra of monofluoro substituted fatty acids measure the orientation along the σ_{11} axis. These differences are accommodated by subdividing the angle β into two angles α and γ . The term γ represents the angle between the molecular interaction vector, \vec{r} , for a specific technique and the instantaneous chain orientation, \vec{c} . The term, α , then represents the angular deviation of the instantaneous chain orientation from the director. The order parameter may therefore be rewritten:

$$\begin{aligned}
 S_{\beta} &= \frac{1}{2} (3\cos^2 \beta - 1) & [28] \\
 &= \left[\frac{1}{2} (3\cos^2 \alpha - 1) \right] \left[\frac{1}{2} (3\cos^2 \gamma - 1) \right] \\
 &= S_{\alpha} S_{\gamma}
 \end{aligned}$$

Under these circumstances, $S_{\alpha} = S_{m01}$. The differences in the geometries of the different molecular interaction vectors are considered in the term S_{γ} . For example, in the all-*trans* conformation the C-D vector is at 90° to the lipid long axis and S_{γ} therefore equals $-1/2$. From this relationship arises the commonly employed transformation:

$$S_{m01} = -2 S_{CD} \quad [29]$$

In certain situations the C-D bond vector is not at 90° with respect to the lipid long axis even in the all-*trans* conformation and S_γ is then no longer $-1/2$. Such situations have been described for deuterons substituted onto a *cis*-double bond (Seelig & Waespe-Sarčević, 1978; Rance *et al.*, 1980) or a cyclopropyl ring (Dufourc *et al.*, 1983; Jarrell *et al.*, 1983). S_γ is often, therefore, referred to as S_{GEO} . An examination of the equations relating the chemical shift anisotropy ($\sigma_{\parallel} - \sigma_{\perp}$) and the ^{19}F -NMR line shape of a monofluoropalmitic acid show that the values of ($\sigma_{\parallel} - \sigma_{\perp}$) are directly modulated through the order parameter S_{11} , corresponding to the order of the long molecular axis. Thus in this case $\gamma = 0^\circ$, $S_\gamma = 1$ and we directly measure S_{m01} , since $S_{11} = S_{m01}$.

There are two immediately apparent mechanisms through which the value of S_{m01} could be modulated. Firstly, if the entire acyl chain were tilted with respect to the director axis, S_{m01} would be reduced at each methylene segment along the chain in proportion to the angle of tilt. Secondly, a single gauche (plus or minus) rotational isomerization at any point along the acyl chain would cause that methylene segment and the succeeding portion of the chain to assume a tilt with respect to the director. Of course, rotational isomerizations occur with high frequency and short lifetimes particularly in the liquid-crystalline state, so that the acyl chains rapidly interconvert between a multitude of possible conformations (i.e., combinations of *trans* and

gauche \pm isomerizations along the chain) and only the average conformation is observed. NMR techniques do not measure the effects of rigid body movements (whole chain tilt) and rotational isomerizations independently so that the measured order parameter contains contributions from both mechanisms.

A major strength of the order parameter formalism lies in the ability to deduce average chain conformations. Since the values of S_{m0} are proportional to the average number of *gauche* \pm versus *trans* conformers at a particular position, it is possible to analyse these data in terms of statistical mechanical models of chain motions in the bilayer. The mean-field, rotational isomerization model of Marcelja (1974) has been successfully applied by Schindler and Seelig (1975) to reproduce S_{m0} values for a bilayer of DPPC, and this model has been further refined by Meraldi and Schlitter (1981a, b) and by Gruen (1982). Such analysis permits the average chain conformation to be described in terms of the probability of there being a *trans* (p_t) versus a *gauche* (\pm) rotational isomer (p_g) at a particular chain position where:

$$p_t + 2 p_g = 1$$

[30]

Petersen and Chan (1977) have evolved equations through which order parameters obtained via different NMR applications relate to the probability of there being a *trans*-conformer. These are:

$$S_{FF} = -3/8 p_i - 1/8 \quad [31]$$

$$S_{CD} = -1/2 p_i \quad [32]$$

$$S_{m01} = 9/8 p_i - 1/8 \quad [33]$$

Note that only when $p_i = 1$ does the common transformation $S_{m01} = -2 S_{CD}$ apply. This is because the presence of a single *gauche* plus or *gauche* minus conformer has different effects on the angle γ between the molecular interaction vector and the instantaneous chain director for different molecular interaction vectors.

IV. THE ^{19}F -NMR SPECTRUM OF A MONOFLUOROPALMITIC ACID IN A LIPID BILAYER

Simulation of the ^{19}F -NMR Spectral Line Shape

Since the ^{19}F -NMR spectrum contains contributions from both chemical shift anisotropic and dipole-dipole interactions, there is no one spectral parameter which is readily interpretable in terms of an orientational order parameter. The alternative is mathematical simulation of the line shape using the model equations described previously. In this section, I will describe the manner in which the chemical shift anisotropy, the intermolecular and intramolecular dipole terms and the order parameter, S_{m01} , interact in the simulated ^{19}F -NMR spectra.

A computer program was first written, which employed the previous mathematical description of the orientation and order dependence of the chemical shift anisotropy and H-F dipole interactions under conditions of axially symmetric motions, to generate a simulation of the ^{19}F -NMR line shape of a monofluoropalmitic acid in a lipid bilayer (Brian McDonough, M.Sc. Thesis, 1981, University of Alberta). The simulated line shape depends on four quantities, the chemical shift anisotropy, the intermolecular dipole broadening, the intramolecular dipole broadening and the order parameter, S_{m01} .

In practice the chemical shift anisotropy was first estimated for a monofluoropalmitic acid chain in the

all-*trans* conformation undergoing axially symmetric rotation about the lipid long axis while aligned perpendicular to the plane of the bilayer. This calculation is described in a subsequent section. This calculated value of $(\sigma_{||} - \sigma_{\perp})$ corresponds to that expected when $S_{mol} = 1$ and is therefore referred to as $(\sigma_{||} - \sigma_{\perp})_{max}$. In the simulation the effective value of $(\sigma_{||} - \sigma_{\perp})$ is never specifically entered but is quantitated through S_{mol} , hence,

$$S_{mol} = \frac{(\sigma_{||} - \sigma_{\perp})_{observed}}{(\sigma_{||} - \sigma_{\perp})_{maximum}} \quad [1]$$

By analogy with the chemical shift anisotropy, the maximum value of the intrachain H-F dipole interaction (Δ_1) was first estimated for a situation in which $S_{mol} = 1$ and subsequently, the effective value of Δ_1 was never considered explicitly other than as a modulation of the maximum value through S_{mol} . Again, the estimation of Δ_1 is described in a later section.

The interchain dipole interaction Δ_0 becomes of significance only in the gel state. In the liquid-crystalline state the rapid lateral diffusion of the lipid molecules drastically reduces the interchain dipole interactions effectively to zero. Since the rates of lipid lateral diffusion decrease as the proportion of gel state lipid increases, the strength of the orientation-independent interchain dipole interactions increase and this effect is accommodated in the simulation by entering increasingly

larger values of Δ_0 (in Hertz).

Figure 4 illustrates the manner in which the values of these various parameters alter the ^{19}F -NMR line shape within the context of the theoretical model. The simulated spectra in row A demonstrate the effects of altering the value of Δ_1 . When Δ_1 is small, the anisotropic chemical shift spectrum, characteristic of rapid long-axis rotation of the entire lipid molecule is obtained. This is the theoretical equivalent of almost complete proton-fluorine decoupling. The sharp perpendicular edges of this spectrum correspond to the quantities $\sigma_{||}$ and σ_{\perp} . The value of $(\sigma_{||} - \sigma_{\perp})_{\text{max}}$ for a monofluoropalmitic acid can be estimated to be 82.2 ppm (Macdonald *et al.*, 1983). Altering the quantity $(\sigma_{||} - \sigma_{\perp})_{\text{max}}$ would change the positions of these inflections without altering the overall line shape. As the value of Δ_1 increases, the anisotropic chemical shift spectrum first broadens and then, when Δ_1 equals approximately 15,000 Hz, assumes the dipolar or "superlorentzian" line shape which resembles that of experimentally obtained ^{19}F -NMR spectra (see later). Increasing the value of Δ_1 by a further order of magnitude extensively broadens the spectrum, particularly in the wings, without altering its "superlorentzian" character.

The spectral simulations in row B of Figure 4 illustrates the effect of increasing the value of Δ_0 . Here, Δ_1 was set to 20,000 Hz so that the overall line shapes would correspond to an experimental situation. Increasing Δ_0

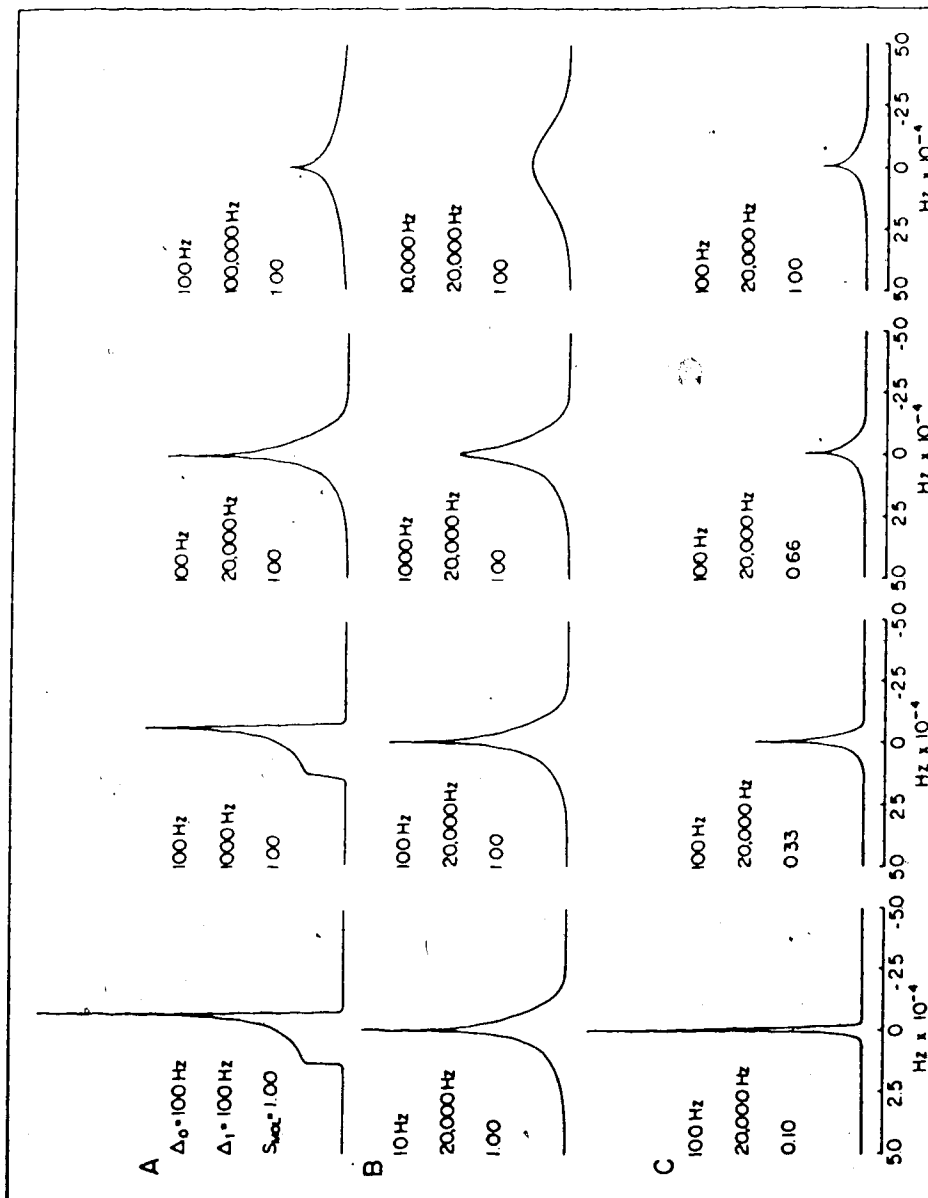


Figure 4: The effect of varying the line shape parameters on the simulated ^{19}F -NMR line shape. A - Variation of the intramolecular dipole parameter Δ_1 ; B - Variation of the intermolecular dipole parameter Δ_0 ; C - Variation of the orientational order parameter S_{mol} .

while holding Δ , small broadens the anisotropic chemical shift spectrum of row A without affecting the overall line shape until Δ_0 becomes excessively large. The effect of increasing Δ_0 on the "superlorentzian" spectrum is to progressively broaden the lines at all frequencies although the effect is most obviously manifest at the "peak". This broadening corresponds to an increase in the efficacy of interchain dipole interactions such as would result from, for example, a transition from the liquid-crystalline to the gel state.

Finally, in row C the manner in which S_{m0} modulates the "superlorentzian" line shape is illustrated. At very low values of S_{m0} , the spectrum approaches the isotropic limit while retaining "superlorentzian" characteristics. As S_{m0} increases, the line shape broadens, particularly in the wings of the spectrum.

Estimation of the Fluorine Chemical Shift Anisotropy

Knowledge of the chemical shielding tensor elements for a monofluoro substituted lipid are required for the interpretation of the ^{19}F -NMR spectra in terms of order parameters. Several different experimental techniques could be used to obtain ^{19}F -NMR chemical shielding information. These include multiple pulse experiments for removing dipolar broadening in order to determine the principal tensor elements (Mehring *et al.*, 1971) in combination with single crystal studies to determine the molecular

orientation of the tensor principal axis system (Griffin *et al.*, 1972). Dissolution of the molecule of interest in nematic liquid crystal solvents to achieve partial alignment (Buckingham & McLaughlan, 1967) or observing the orientation dependence of the resonance frequency in macroscopically oriented bilayers (Engelsberg *et al.*, 1983) can be used to estimate effective chemical shift anisotropies. Alternately, Burnell *et al.* (1981) report a method of determining the complete chemical shift tensor from the field dependence of the moments of the magnetic resonance line shape.

The strength of the $^1\text{H} - ^{19}\text{F}$ dipolar interaction and the proximity of ^1H and ^{19}F resonance frequencies prevent a straight forward determination of the chemical shift anisotropy of a monofluoropalmitic acid. The chemical shift tensor elements of a $-\text{CH}_2-\text{CHF}-\text{CH}_2-$ type fluorine in its principal axis system (P.A.S.) have yet to be determined. Each of the aforementioned techniques present unique experimental difficulties or require apparatus beyond that available. As such I have relied instead upon a value of the effective chemical shift anisotropy estimated from known quantities determined for teflon.

For the measured traceless chemical shift anisotropy tensor in teflon (polytetrafluoroethylene), three distinct elements were found: $\sigma_{11} = -80$ ppm, $\sigma_{22} = +21$ ppm and $\sigma_{33} = +59$ ppm (Mehring *et al.*, 1971). The direction of the principal tensor axes for CF_3COOAg were also described and later confirmed in a single-crystal study (Griffin *et al.*,

1972). The most shielded element, σ_{33} , was aligned along the C-F bond and the least shielded component, σ_{11} , was perpendicular to the 33 axis in the C-C-F plane. This is considered to be the principal axis system for the fluorine nucleus in a monofluoropalmitic acid and is illustrated in Figure 2. Rotation of this axis system by 30° about the 33 axis realigns the principal axis system into a new molecule-fixed axis system with an axis parallel to the bilayer normal (see Figure 2). This transformation is accomplished by means of orthogonal rotation matrices, R_{ij} .

$$\sigma' = R^{-1} \sigma_{pas} R \tag{2}$$

Here $R \cdot R^{-1} = R^{-1} \cdot R = 1$ since the Hamiltonian must be invariant to orthogonal coordinate transformation. Since σ_{pas} is diagonalized in its principal axis system, it has the form:

$$\sigma_{pas} = \begin{vmatrix} \sigma_{xx} & 0 & 0 \\ 0 & \sigma_{yy} & 0 \\ 0 & 0 & \sigma_{zz} \end{vmatrix} \tag{3}$$

Following the format of Van *et al.* (1974), any one generalized coordinate system (A,B,C) may be rotated into another (a,b,c) by three successive rotations corresponding to the Euler angles ϕ , θ , and ψ . The generalized transformation matrix of direction cosines is:

	A	B	C
a	$\cos\theta\cos\phi\cos\psi - \sin\phi\sin\psi$	$\cos\theta\sin\phi\cos\psi + \cos\phi\sin\psi$	$-\sin\theta\cos\psi$
b	$-\cos\theta\cos\phi\sin\psi - \sin\phi\cos\psi$	$-\cos\theta\sin\phi\sin\psi + \cos\phi\cos\psi$	$\sin\theta\sin\psi$
c	$\sin\theta\cos\phi$	$\sin\theta\sin\phi$	$\cos\theta$

Choosing $\phi = 0^\circ$, $\theta = 30^\circ$ and $\psi = 0^\circ$ the generalized matrix reduces to that specific for a rotation of 30° about the C-F bond axis,

$$R = \begin{bmatrix} \cos\theta & 0 & -\sin\theta \\ 0 & 1 & 0 \\ \sin\theta & 0 & \cos\theta \end{bmatrix} \quad [4]$$

$$R^{-1} = \begin{bmatrix} \cos\theta & 0 & \sin\theta \\ 0 & 1 & 0 \\ -\sin\theta & 0 & \cos\theta \end{bmatrix} \quad [5]$$

multiplying the three matrices, the resulting matrix is

$$\sigma'_{ij} = \begin{bmatrix} (\cos^2\theta_{xx} + \sin^2\theta_{zz}) & 0 & (-\cos\theta\sin\theta_{xx} + \cos\theta\sin\theta_{zz}) \\ 0 & \sigma_{yy} & 0 \\ (-\cos\theta\sin\theta_{xx} + \cos\theta\sin\theta_{zz}) & 0 & (\sin^2\theta_{xx} + \cos^2\theta_{zz}) \end{bmatrix} \quad [6]$$

These represent the values of the fluorine chemical shift tensor elements in the new molecule-fixed axes system.

Upon introducing the rotational time averaging relevant to the lipid bilayer, I will follow the format of Post *et al.* (1982). For any process of rotational time averaging, the averaged chemical shift tensor assumes the form:

$$\sigma'_{ij} = \left\{ \overline{A\sigma'(1,2,3)A^{-1}} \right\}_{ij} = \sum_{k,l} \sigma'(1,2,3)_{kl} \overline{(a_{ik}a_{jl})} \quad [7]$$

where a_{ik} and a_{jl} are the direction cosines. In the first order the relevant term is σ'_{zz} , which can be written as:

$$\begin{aligned} \sigma'_{zz} &= \sum_{k,l} \sigma'(1,2,3)_{kl} \overline{(a_{zk}a_{zl})} \\ &= \sum_{kl} \sigma'(1,2,3)_{kl} (a_{zk}a_{zl} \frac{1}{3}\delta_{kl}) + \frac{1}{3} \sum_{kl} \sigma'(1,2,3)_{kl} \delta_{kl} \\ &= \frac{2}{3} \sum_{kl} \sigma'(1,2,3)_{kl} S'_{kl} + \bar{\sigma} \end{aligned} \quad [8]$$

where S_{kl} are now the order parameters with respect to the magnetic field and $\bar{\sigma} = 1/3 \sum_k \sigma_{kl}$ is the isotropic shielding factor. The values of δ_{kl} are the Kronecker deltas. The anisotropy of the spectrum can also be written as:

$$\sigma'_{zz} = \frac{2}{3} \sum_{kl} (1,2,3)_{kl} S_{kl} - \frac{1}{2} (3\cos^2\theta - 1) \quad [9]$$

where S_{kl} are now the order parameters with respect to the axis of motional averaging and θ is the angle between the axis of motional averaging and the magnetic field, as previously. If we allow $\Delta\sigma$ to equal the width of the chemical shift distribution function for σ'_{zz} , then:

$$\Delta\sigma = \left| \sum_{kl} \sigma'_{kl} (1,2,3)_{kl} S_{kl} \right| \quad [10]$$

Note again that

$$S_{kl} = \frac{1}{2} \frac{(\cos\beta_k \cos\beta_l - \delta_{kl})}{(3\cos\beta_k \cos\beta_l - \delta_{kl})} \quad [11]$$

where $k, l = 1, 2, 3$ for x, y, z of the molecule-fixed coordinate system and β_k is the angle between the k th axis and the axis of motional averaging. Using the indices of Figure 2, it can be ascertained that S_{xz} and S_{zx} are zero by symmetry, while S_{yz} might have a small value. Assuming that S_{yz} is negligible compared to the diagonal elements so that x, y and z are the principal axes of S , one can now write:

$$\sigma'_{xx} S_{xx} + \sigma'_{yy} S_{yy} + \sigma'_{zz} S_{zz} \quad [12]$$

Invoking the conditions of axially symmetric motions the distribution function becomes

$$\Delta\sigma = S_{zz} (\sigma_{\perp} - \sigma_{\parallel}) \quad [13]$$

where

$$\sigma_{\parallel} = (\sigma'_{zz}) \quad [14]$$

and

$$\sigma_{\perp} = 1/2(\sigma'_{xx} + \sigma'_{yy}) \quad [15]$$

Evaluating these quantities from the values of the fluorine chemical shift tensor elements in the new molecule-affixed axis system, one obtains:

$$\sigma'_{xx} = (\cos^2\theta\sigma_{xx} + \sin^2\theta\sigma_{zz}) = -4.25 \text{ ppm}$$

$$\sigma'_{yy} = \sigma_{yy} = +59.00 \text{ ppm}$$

$$\sigma'_{zz} = (\sin^2\theta\sigma_{xx} + \cos^2\theta\sigma_{zz}) = 54.75 \text{ ppm}$$

and,

$$\sigma_{\parallel} = -54.75 \text{ ppm}$$

$$\sigma_{\perp} = 27 \text{ ppm}$$

$$(\sigma_{\parallel} - \sigma_{\perp})_{\text{max}} = -82.2 \text{ ppm}$$

In the absence of a direct experimental evaluation of the chemical shift tensor elements of a monofluoro-type fluorine and its principal axis system, or of a measurement of the effective chemical shift anisotropy of a monofluoro fatty acid in a lipid bilayer, I have consistently used the above estimate of $(\sigma_{\parallel} - \sigma_{\perp})_{\text{max}}$ in the fluorine line shape simulations throughout these studies.

Several points concerning the accuracy of this estimate require further discussion. The values of the chemical shift tensor elements in the principal axes system were those reported by Mehring *et al.* (1971) for teflon. Although there is no reason to assume *a priori* that a monofluoro substituted species should have tensor components identical in value to those of teflon, an examination of the tensor components determined by Mehring *et al.* (1971) for a range

of different fluorine-substituted species shows that the deviations are not great. Secondly, it has been assumed the principal axis system defined for CF_3COOAg corresponds to that of a monofluoro fatty acid. Choosing another principal axis system such as described by Post *et al.* (1982) results in a different value for $(\sigma_{||} - \sigma_{\perp})_{\text{max}}$. Thirdly, since we define:

$$S_{\text{mol}} = \frac{(\sigma_{||} - \sigma_{\perp})_{\text{obs}}}{(\sigma_{||} - \sigma_{\perp})_{\text{max}}}$$

the accuracy of the orientational order parameters obtained by simulation of the experimental ^{19}F line shape rest squarely on the accuracy of the estimated value of $(\sigma_{||} - \sigma_{\perp})_{\text{max}}$. If this quantity was either a significant over- or underestimate, the extracted values of S_{mol} would be inversely under- or over estimated, respectively.

Estimation of the Intrachain Fluorine-Proton Dipolar Interaction

The value of the maximum intrachain H-F dipolar interaction has been estimated experimentally by a study of the field dependence of the ^{19}F -NMR line shape of monofluoropalmitic acids biosynthetically incorporated into membranes of *A. laidlawii* B (Macdonald *et al.*, 1983; B. McDonough, M.Sc. Thesis, University of Alberta, 1981). In this study use was made of the fact that dipolar broadening is independent of H_0 , whereas the Hamiltonian describing the

chemical shifts is linear in H_0 . Given an estimate of $(\sigma_{\parallel} - \sigma_{\perp})_{\max}$, and employing conditions in which S_{m0} was equal at each field strength, a consistent value of $\Delta_1 = 20,000-25,000$ Hz was found adequate to simulate the $^{19}\text{F-NMR}$ spectra obtained at a number of field strengths.

In this section I will describe calculations which indicate how the value of Δ_1 is obtained theoretically and that the experimental and theoretical values are in substantial agreement. As noted previously, the tensors for dipole-dipole interactions scale as $(3\cos^2\theta - 1)$, where θ is the angle between the axis of motional averaging and the magnetic field. The individual F-H dipole interactions may be represented by a vector \vec{n} which is directed along the line joining the two nuclei under consideration and whose size is proportional to (in units of angular frequency)

$$\vec{n} = \gamma_H \gamma_F \hbar / r_{\text{H-F}}^3 \quad [16]$$

where γ_H and γ_F are the magnetogyric ratios of the proton and fluorine, respectively, and $r_{\text{H-F}}$ is the proton-fluorine internuclear distance. Under conditions of axially symmetric motions, it is the projection of the vector \vec{n} onto the axis of motional averaging that scales according to $(3\cos^2\theta - 1)$, so that one may write:

$$\Delta_{1ij} = \frac{\gamma_{\text{H}^1\text{F}} \hbar}{r_{ij}^3} \cdot \frac{1}{2} (3\cos^2\beta_{ij} - 1) \quad [17]$$

where β_{ij} is the angle between the internuclear vector \vec{r}_{ij} and the axis of motional averaging. The total intrachain H-F dipolar interaction is then:

$$\Delta_1 = \sum_{ij} |\Delta_{1ij}| \quad [18]$$

where the summation is over all H-F pairs.

Figure 5 illustrates the structure of a monofluoro substituted fatty acid in the all-*trans* conformation and the calculated internuclear distances. These values were obtained by assuming tetrahedral bond angles and C-C, C-H and C-F bond lengths of 1.541 Å, 1.094 Å and 1.32 Å, respectively. Internuclear distances of greater than 4 Å were not considered because the $1/r^3$ dependency would make Δ vanishingly small. In Table 1, the calculated values are categorized according to the length of the H-F internuclear vector. The number of H-F pairs per radius category is listed in brackets. When summed over all Δ_{ij} , the theoretical value of 28,270 Hz is obtained for the total intrachain F-H dipolar interaction of a monofluoropalmitoyl chain in an all-*trans* conformation undergoing axially symmetric rotations. This theoretical value is in good agreement with the value of Δ_1 obtained experimentally (Macdonald *et al.*, 1983) when the approximations of the calculations (bond

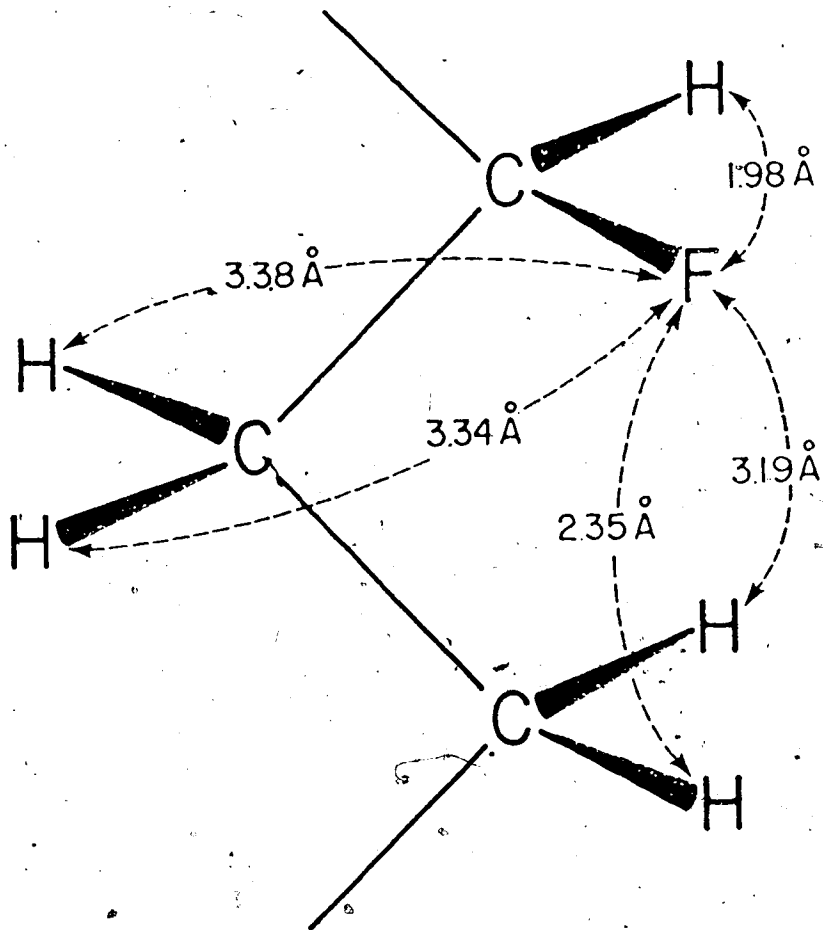


Figure 5: Geometry of the H-F intramolecular dipolar interactions.

Table 1: F-H Intra-chain Dipolar Interaction Parameters

r (Å) [x]	$\bar{\nu}_{1j}$ (rad-sec ⁻¹)	$\bar{\nu}_{1j}$ (Hz)	θ_{1j}	δ (3cos ² θ_{1j} -1)	ν_j (Hz)
1.98 [1]	9.12×10^4	14,500	30°	-0.500	1,250
2.53 [2]	4.37×10^4	6,950	58°	0.388	1,250
3.20 [2]	2.16×10^4	3,430	34°	0.530	1,800
3.35 [2]	1.88×10^4	2,980	69°	-0.307	1,100
3.38 [2]	1.83×10^4	2,900	70°	-0.324	1,100

* See text for explanations of symbols

lengths and angles) are considered.

It is interesting to note that the greatest effective dipolar interactions appear to be between the geminal proton and the fluorine as expected, and between the fluorine and protons bonded to the carbon at the beta position relative to the fluorine-substituted carbon rather than the alpha bonded protons. There is some experimental evidence which supports this theoretical conclusion. Engelberg *et al.* (1983) studied the ^{19}F -NMR line shapes of *geminal*-difluoro substituted fatty acyl containing lipids and observed little change in the effective H-F dipolar interactions when deuterons were substituted for protons on the vicinal carbons. If the effective vicinal H-F dipolar interactions were extensive, then a substantial narrowing of the ^{19}F spectrum and a closer approximation of the predominantly anisotropic chemical shift spectrum would be expected upon substitution of deuterons for protons.

The Assumption of Effective Axial Symmetry

The validity of the spectral simulation employed throughout these studies, and hence of the extracted values of the orientational order parameters, rests upon the assumption that the lipid molecules are rotating about their long axes at a rate sufficient to produce effective axial symmetry. The assumption of effective axial symmetry requires that the rate of long axis rotation of the entire lipid molecule be faster than the timescale of the

interaction being measured, τ , where δ is for chemical shift anisotropy (Davis, 1983)

$$\tau = 1/\gamma H_0 \Delta\sigma$$

[19]

It is expected that upon the transition from the liquid-crystalline state to the gel state the rates of long axis rotation of individual lipid molecules should be severely attenuated. Studies by Marsh (1981) using nitroxide spin-labelled lipids indicate that, as the temperature is lowered through the lipid phase transition, there is an abrupt decrease in the rates of rotational diffusion at T_m followed by a further gradual decrease in rotational rates at lower temperatures. While one might argue that the perturbing effect of the bulky nitroxide spin labels prevents using such data quantitatively, nevertheless the qualitative picture is probably valid. Therefore, at some temperature either at or below the lipid phase transition, the assumption of axial symmetry will no longer be valid.

Although the ^2H -NMR line shape of specifically deuterated lipids apparently reflects axially symmetric motions of the lipid molecules when obtained in the liquid-crystalline state, at temperatures at or below the lipid phase transition a gel state component appears in the spectrum which clearly can no longer be ascribed to axially symmetric motions. In the deuterium case

$\tau(^2\text{H}) \cong 1.3 \times 10^{-8}$ sec (Davis, 1983). In contrast, the line

shape obtained via ^{13}C -NMR of DPPC, which was ^{13}C enriched at the C-1 position of the sn-2 chain, was still indicative of axially symmetric motions of the lipid molecules even at a temperature approximately 40°C below the lipid phase transition (Wittebort *et al.*, 1981), where the timescale of measurement was $\tau(^{13}\text{C}) \cong 2.0 \times 10^{-5}$ sec (Davis, 1983). The rotational correlation time of the lipid itself at temperatures below the phase transition must then lie somewhere between $\tau(^2\text{H})$ and $\tau(^{13}\text{C})$.

The timescale of measurement for ^{19}F , assuming a maximum chemical shift anisotropy of 82.2 ppm and at 254.025 MHz, would be $\tau(^{19}\text{F}_{\text{CSA}}) \cong 7.6 \times 10^{-6}$ sec. Thus, it is to be expected that the assumption of axial symmetry should hold for the ^{19}F -NMR spectra of a monofluoropalmitic acid in the gel state at temperatures lower than for ^2H -NMR of deuterated lipids but not so low as for ^{13}C -NMR.

For the case of heteronuclear dipole-dipole interactions, which also contribute to the ^{19}F line shape, in order to project the dipolar interaction vectors, n , onto the axis of motional averaging, the rates of rotational diffusion of the lipid molecules must be on a timescale which is short compared to the inverse of the static interaction $\tau(^{19}\text{F}_{\text{DD}})$ and

$$\tau(^{19}\text{F}_{\text{DD}}) \cong (\gamma_{\text{H}}\gamma_{\text{F}}h/2\pi r_{\text{HF}}^3)^{-1} \quad [20]$$

where the symbols retain their earlier definitions (Farrar

and Becker, 1971). From Table 1 the strongest static F-H dipolar coupling is between fluorine and the geminal proton and equals approximately 14,500 Hz. Therefore $\tau(^1F_{DD}) \cong 6.8 \times 10^{-9}$ sec, indicating that the assumption of rapid long-axis rotational reorientation should pertain to the case of intramolecular fluorine-proton dipolar interactions at temperatures below the lipid phase transition as low as those at which the ^{13}C spectra still reflect axially symmetric motions.

Figure 6 compares the ^{19}F -NMR spectrum of a solid crystalline powder of 12F16:0 with that of membranes of *A. laidlawii* B B enriched with 20 mole % 12F16:0 plus 80% 16:0 in the gel state. The two spectra were obtained under identical instrumental conditions. It is expected that in the absence of motions rapid enough to average the chemical shift and dipolar interactions, the ^{19}F spectrum should broaden in proportion to the values of the unaveraged chemical shift tensor elements (estimated to be: $\sigma_{11} = -80$ ppm, $\sigma_{22} = 21$ ppm, $\sigma_{33} = 59$ ppm, by comparison with the model compound teflon, Mehring *et al.*, 1971). Further broadening due to fluorine-proton dipolar interactions would effectively obscure the inflections corresponding to the values of the individual chemical shift tensor elements resulting in the approximately Gaussian line shape of the solid 12F16:0 in Figure 6. The ^{19}F -NMR spectrum of 12F16:0 incorporated into membranes of *A. laidlawii* B in the gel state exhibits none of the spectral characteristics

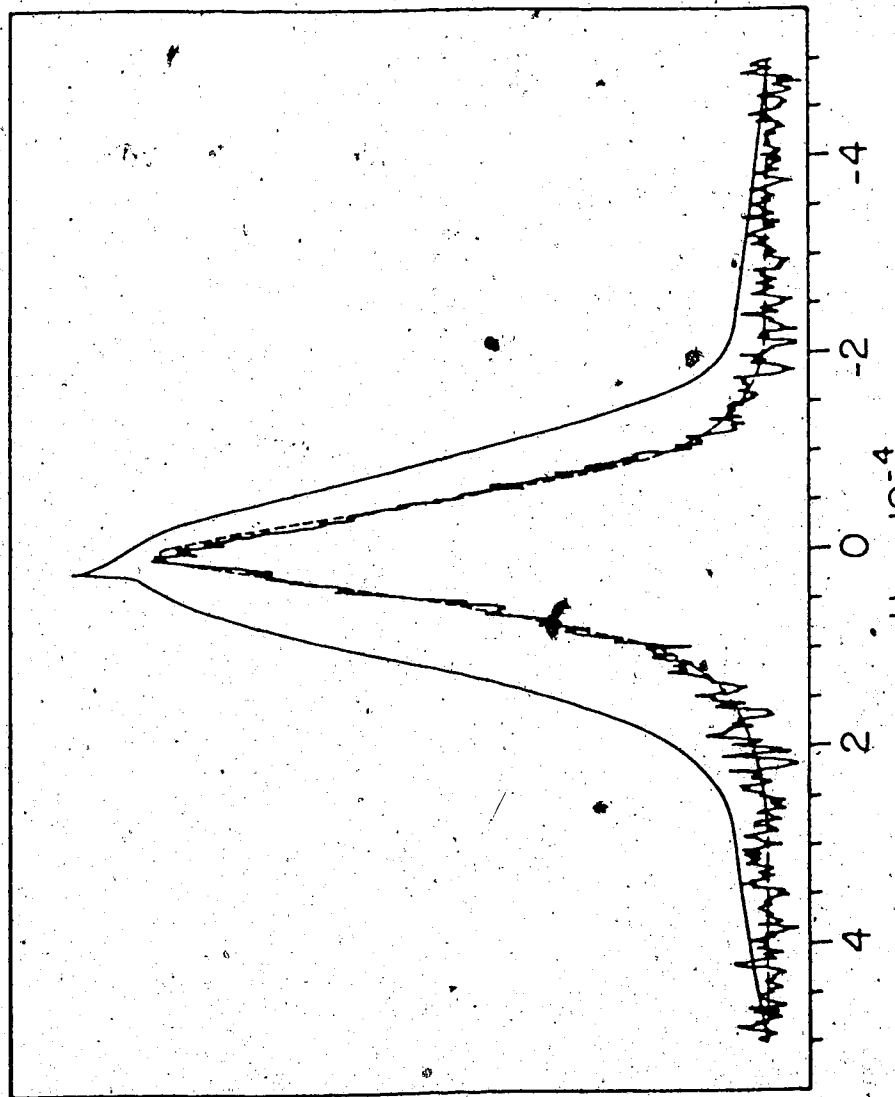


Figure 6: ^{19}F -NMR spectra of solid 12F16:0 (dashed line, 1000 scans) and A. laidlawii B membranes enriched with 20 mole % 12F16:0 plus 80 mole % 16:0 at 279°K (solid line, 100,000 scans). The simulated spectrum ($S_{\text{mol}}=0.82$; $\Delta_0=1500$ Hz, $\Delta_1=20,000$ Hz) is superimposed.

of axial asymmetry which are observed at extremely low temperatures when ^{13}C -NMR (Wittebort *et al.*, 1981) or ^{31}P -NMR (Campbell *et al.*, 1979) lipid spectra are investigated. The gel-state spectrum is considerably narrowed relative to the solid spectrum, indicating that significant motional averaging occurs on the ^{19}F -NMR time-scale. Furthermore, the gel state spectral line shape is "superlorentzian", an indication that the motions which average the chemical shift and dipolar interactions are axially symmetric in nature. Finally, the mathematical model of the ^{19}F -NMR line shape, which assumes axially symmetric motions of the lipid molecules, adequately simulates the experimentally obtained spectrum in the gel state as shown in Figure 6. Here the spectrum of 12F16:0 was acquired at 279°K, almost 40°K below the corresponding membrane lipid phase transition. S_{mol} was found to be 0.82, indicating that the fatty acyl chain was approaching the all-*trans* configuration. Δ_0 , the interchain interaction indicator, was approximately 1500 Hz, demonstrating that the limit in which the "superlorentzian" line shape is obtained ($\Delta_0 \ll \Delta_1$ or $\Delta_0 \ll \Delta\sigma$) was readily met at these temperatures (Wennerstrom and Lindblom, 1977).

Many more examples of the correspondence of experimental and simulated ^{19}F -NMR spectra will be provided throughout the remainder of this thesis. However, few cases provide such an opportunity to test the limits of the assumptions which have been made in order to extract

information from the $^1\text{F-NMR}$ spectra. The theoretical considerations described above, the "superlorentzian" character of the gel state $^1\text{F-NMR}$ spectrum and the successful simulation of that spectrum using an "axially symmetric" mathematical model all suggest that the orientational order of the MRPA probe may be described in both the gel state and in the liquid-crystalline state in terms of a single orientational order parameter, S_{m01} .

The $^1\text{F-NMR}$ Spectrum in a Region of Mixed Lipid Phases

The phase transitions of various model and natural membranes have been extensively investigated using $^1\text{H-NMR}$. Recently, attempts have been made to measure the fractions of fluid and gel-phase components present during the course of the phase transition. Such information has been obtained directly from the ^1H spectrum by taking the ratio of the estimated spectral area of the fluid component to the total spectra area (Nichol *et al.*, 1980). Alternately, the fraction of fluid phase was determined by simulating the broad component of the ^1H spectra (Kang *et al.*, 1979; 1981). Jarrell *et al.*, (1981) demonstrated that the $^1\text{H-NMR}$ spectral moments could be utilized to analyze the composition of mixed lipid phases.

Each of these techniques of spectral analysis assume that the gel and liquid-crystalline phases are in slow exchange on the $^1\text{H-NMR}$ timescale, so that the $^1\text{H-NMR}$ spectrum in the phase transition region is a simple

superposition of gel and liquid-crystalline state spectra. Therefore the n th moment of the ^1H spectrum of a mixture of gel and liquid-crystalline phases is given as

$$M_n = fM_n^L + (1-f)M_n^G \quad [21]$$

where f is the fraction of lipid in the fluid phase, and M_n^L and M_n^G are the n th moments of the fluid and gel components of the spectrum, respectively (Nichol *et al.*, 1980; Jarrell *et al.*, 1981). Implicit in this expression is the assumption that M_n^L and M_n^G remain essentially constant over the temperature range of the phase transition.

The same conditions should apply in the case of ^{19}F -NMR spectra in the region of the phase transition, since gel- and fluid-phase components should be in slow exchange on the ^{19}F -NMR timescale. In this section I will describe results which indicate that the ^{19}F -NMR spectrum is a simple superposition of gel-phase and liquid-crystalline-phase spectra and that the fraction of fluid or gel component required to simulate spectra acquired with mixed phases corresponds to the fraction of fluid or gel component estimated from DSC of the membrane lipids.

Figure 7 shows ^{19}F -NMR spectra of *A. laidlawii* B membranes enriched with 80 mole % 19:0cp tΔ9 plus 20 mole % 10F16:0. These spectra were normalized with respect to the number of acquisitions required to obtain the spectra by scaling with a factor K where:

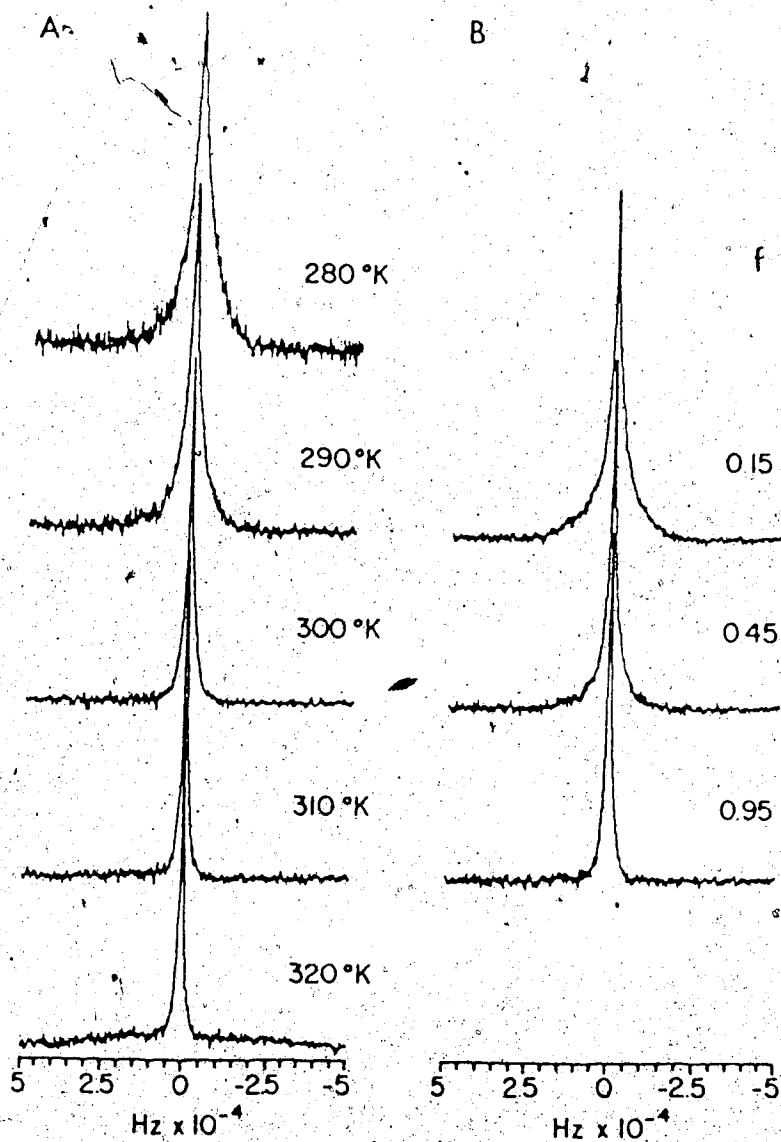


Figure 7: The ^{19}F -NMR spectrum in a region of mixed phases as a superposition of gel state and liquid-crystalline state spectra. A - ^{19}F -NMR spectra of A. laidlawii B membranes enriched with 80 mole % 19:0cp,tA9 plus 20 mole % 10F16:0 after normalization to the number of scans acquired; B - ^{19}F -NMR spectra simulated by combining spectra obtained at 280 °K and 320 °K according to the indicated fractions (f is for the liquid-crystalline phase fraction).

$$K = 5000/\text{no. of acquisitions} \quad [22]$$

since the least number of acquisitions acquired in this series of spectra was 5000. Following this normalization procedure each spectrum had an equivalent integrated peak area. Similarly, the ratio of the baseline noise in any two spectra A and B corresponded to:

$$\frac{\text{noise A}}{\text{noise B}} = \frac{\sqrt{\text{no. of acquisitions A}}}{\sqrt{\text{no. of acquisitions B}}} \quad [23]$$

after normalization with the factor K.

DSC of the corresponding *A. laidlawii* B membrane lipids indicated that the ^{19}F -NMR spectrum acquired at 280°K should correspond to 100% gel phase while that acquired at 320°K should correspond to 100% liquid-crystalline phase. (The DSC endotherms obtained in this situation are fully described in Chapter 5.) The simulated spectra obtained by adding together the 100% gel and 100% liquid-crystalline spectra in the proportions which resulted in the closest simulation of the actual ^{19}F -NMR spectra acquired at 290, 300 and 310°K (i.e. in the region of the phase transition) are shown in Figure 7. The fraction, f , of the 100% liquid-crystalline spectra which was required for the particular simulation is indicated. It is evident from Figure 7 that the extensive broadening of the ^{19}F spectrum which accompanies a decrease in temperature and a transition to the gel state is

effectively reproduced by adding together 100% gel and 100% fluid spectra in the proper proportion. Furthermore, the simulated spectra are indistinguishable from the actual ^{19}F spectra. Therefore, as with ^1H -NMR, the ^{19}F spectra in regions of mixed phases can be considered a simple superposition of gel-state and liquid-crystalline-state spectra.

In Figure 8 the calculated fraction of liquid-crystalline phase lipid, f , obtained by spectral simulation is compared with the % transition of the corresponding *A. laidlawii* B membrane lipids obtained via DSC. The % transition data expresses the integral of the enthalpy of the lipid phase transition as a function of temperature and is, in fact, a measure of the % gel phase (or % fluid phase) lipid at a particular temperature. It is apparent from Figure 8 that both the quantity f and the % transition behave as identical functions of temperature. Thus the ^{19}F -NMR spectrum can be considered a superposition of gel-phase and fluid phase spectra, and the change in the proportion of these two components with temperature accurately reflects the progress of the lipid phase transition as detected calorimetrically.

Relaxation Time Measurements

Since molecular ordering and motional correlation times are really two sides of the same motional coin, it was of interest to measure the ^{19}F -NMR longitudinal relaxation times (T_1) of the monofluoro substituted fatty acyl nuclear

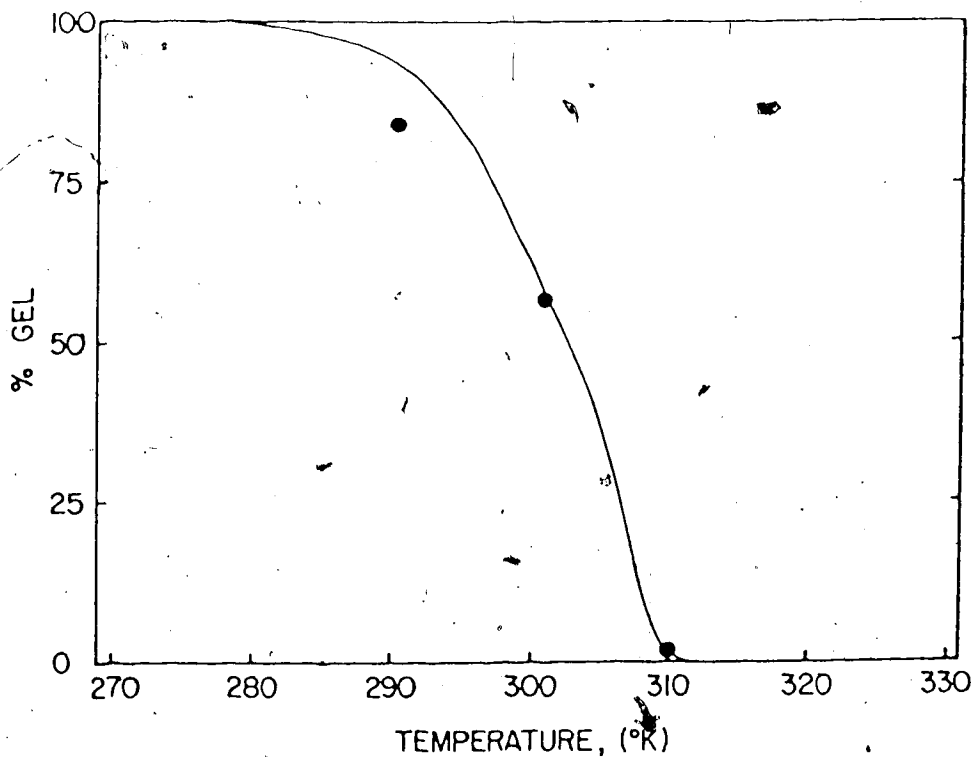


Figure 8: A comparison of the fraction of liquid-crystalline phase lipid calculated calorimetrically with that estimated by simulation of the ^{19}F -NMR spectra. The % transition data were obtained by integration of the enthalpy of the transition endotherm obtained in the case of *A. laidlawii*-B membrane lipids enriched with 80 mole % 19:0cp,t49 plus 20 mole % 10F16:0 by DSC. The fraction of liquid-crystalline phase lipid (closed circles) was estimated from simulation of ^{19}F -NMR spectrum, as described for Figure 7.

spin probes. Figures 9 and 10 show two representations of the type of data obtained using the inversion recovery pulse sequence described in the methods, in this case for A.

laidlawii B membranes enriched with 15 mole % 10F16:0 plus 85 mole % 18:1 cΔ4 at 310°K. The delay times, τ , (D_1) between the inversion pulse and the sampling pulse are indicated for the appropriate spectrum. Using the data of Figure 10, and employing the usual $\ln(S_0 - S_T)$ vs τ plot to obtain T_1 , indicated that the nuclei relaxed at approximately the same rates over the width of the entire spectrum. This point is evident just from an examination of Figure 10. In Figure 11, the values of S_T^* and of $\ln(S_0 - S_T)$ are plotted versus the delay time τ . The value of S_0 was estimated using a three-parameter data-reduction routine based on the report of Levy and Peat (1975). This analysis includes considerations of imperfect r_f pulse lengths and of the pulse sequence interval, D_5 . While the inversion-recovery method is relatively insensitive to imperfect pulse lengths (Levy and Peat, 1975), the D_5 value should be set such that $D_5 \geq 4$ times the longest T_1 measured. Failure to meet this condition will result in an under-estimation of the T_1 values. However, the data reduction routine compensates for such effects. Specifically, the data are fit to:

$$M_2^t = \frac{M_0 \{ (1 - e^{-t/T_1}) + k(1 - e^{-D_5/T_1})e^{-t/T_1} \}}{(1 - k e^{-D_5/T_1})e^{-t/T_1}} \quad [24]$$

where D_5 is the recycle time and

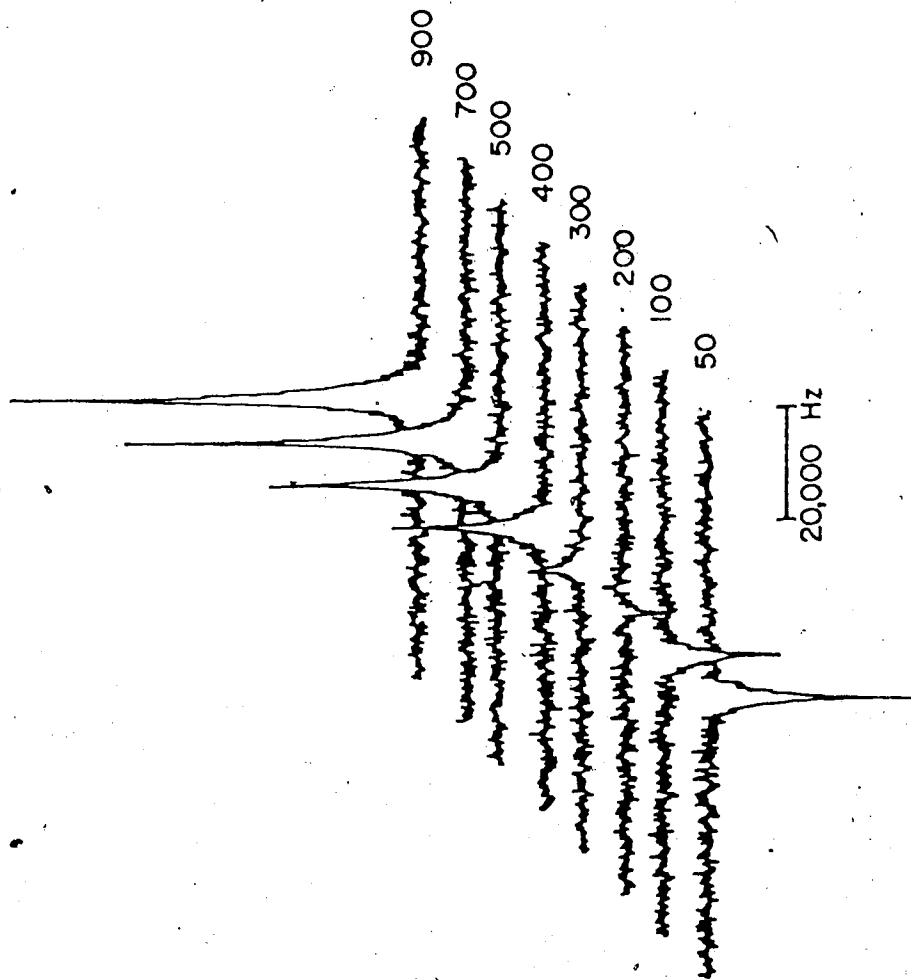


Figure 9: Stacked plot of ^{19}F -NMR spectra obtained using the inversion recovery technique. The sample was A. laidlawii B membranes enriched with 15 mole % 10F16:0 plus 85 mole % 18:1c Δ 4 at 310 $^\circ$ K. The delay times $\tau(D_1)$ are indicated in milliseconds.

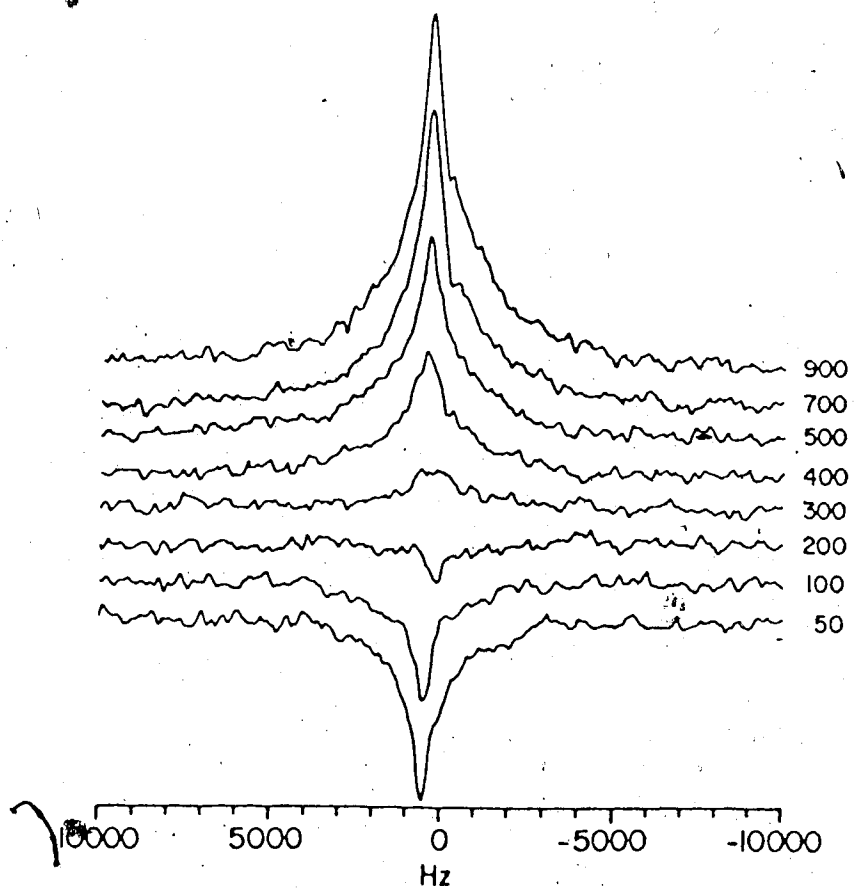


Figure 10: Expanded plot of ^{19}F -NMR spectra obtained using the inversion recovery technique. The sample was the same as in Figure 7 and the delay times $\tau(D_2)$ are indicated in milliseconds.

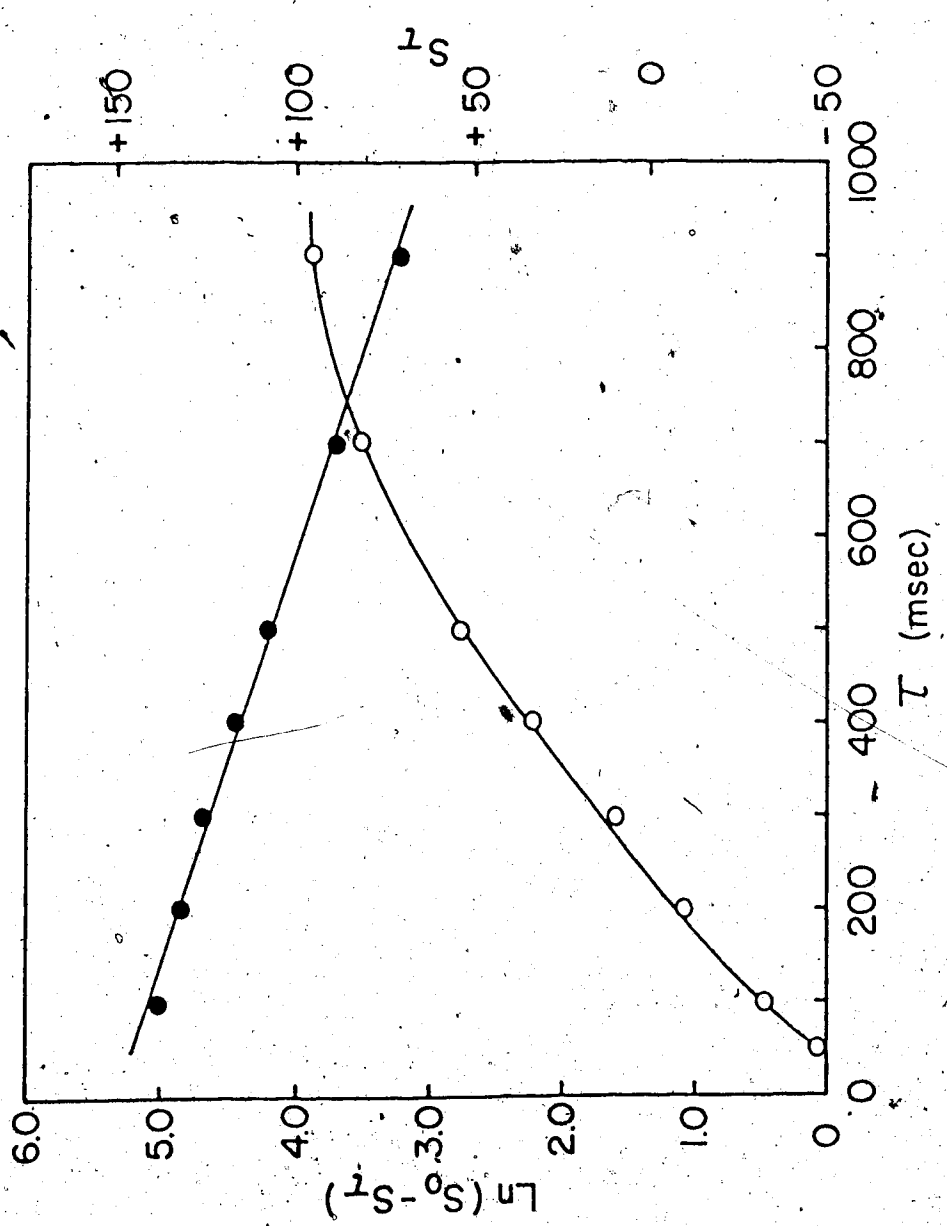


Figure 11: Measurement of the fluorine T1 relaxation time. Open circles, S_t ; closed circles, $\ln(S_0 - S_t)$.

$$k = \sin^2\phi + \cos\psi\cos^2\phi \quad [25]$$

ϕ is the angle which H_{EFF} makes with respect to the axis along which H_1 is applied, and

$$\psi = \gamma H_{\text{EFF}} \tau = \alpha / \cos\phi \quad [26]$$

where τ is the pulse length and α is the flip angle ($\gamma H_1 \tau$). Under optimum conditions (i.e. $k = -1$, $k' = 0$, $D_5 > 4 T_1$), equation 24 reduces to the familiar

$$M_2' = M_0 [1 - 2e^{-t/T_1}] \quad [27]$$

Table 2 lists the T_1 values estimated for different positional isomers of MFPA in the presence of 18:1 cΔ4 at 310°K. It is clear that a gradient of T_1 values exists with the shorter T_1 's (slower motions) nearest the carbonyl head-group and the longer T_1 's (faster motions) nearest the methyl terminus of the fatty acyl chain. This is the same qualitative picture of the variation in relaxation times along the fatty acyl chain which is obtained via ^{13}C -, ^2H -, and ^1H -NMR techniques (e.g., see Jardetzky and Roberts, 1982). A quantitative interpretation of these ^{19}F -NMR relaxation measurements in terms of relaxation mechanisms and motional correlation times would require more accurate and systematic relaxation measurements.

Table 2: ^{19}F -NMR Longitudinal Relaxation Times (T_1) and Order Parameters (S_{mol}) of *A. laidlawii* B Membranes Enriched with 15 mole % xF16:0 plus 85 mole % 18:1c44.

MFPA	T_1 (msec)	S_{mol}
6F16:0	445	0.23
8F16:0	435	0.20
10F16:0	444	0.20
12F16:0	624	0.16
14F16:0	770	0.12

In summary, the ^{19}F -NMR spectra of a MFPA can be simulated using a model which assumes effectively axially symmetric motions of the lipid molecules in a lipid bilayer. Via reasonable assumptions concerning the effective values of the important interactions, the chemical shift anisotropy and the intrachain H-F dipole-dipole interactions, under conditions of axially symmetric motions, these simulations can yield information concerning the orientational ordering of the fatty acid long axis. Moreover, it would appear on the basis of both theoretical and experimental considerations that a single order parameter adequately describes the ^{19}F -NMR spectrum in both the liquid-crystalline and gel phases. It can be further demonstrated that the ^{19}F -NMR spectra faithfully reflect the progress of the lipid phase transition and provide at least the same qualitative picture of fatty acyl chain ordering and dynamics as obtained from ^2H -NMR.

V. ORIENTATIONAL ORDER AND FATTY ACID STRUCTURE

A Straight-Chain Saturated Fatty Acid

In the remaining portions of this thesis I will describe the results of investigations into the effects of different classes of fatty acyl structural substituents on the orientational ordering of the monofluoropalmitoyl nuclear spin probes in membranes of *A. laidlawii* B. The strength of these studies resides in the coupling of spectroscopic analysis ($^{19}\text{F-NMR}$) with an independent determination of the physical state of the membrane lipids (DSC). This combination permits the relationships between fatty acyl structure, orientational order and the lipid phase transition to be queried. In order to establish a basis for comparison with fatty acids of more complex structure, the orientational order parameters of the MFPA's were first determined in the presence of the straight-chain, saturated fatty acid 16:0.

In these experiments with 16:0 enrichment, the following protocol was followed. Whole cells from 1.0 litre of culture medium (supplemented with 20 mole % MFPA plus 80 mole % 16:0) were washed once with β -buffer and, after resuspension in β -buffer containing 90% deuterium oxide, the $^{19}\text{F-NMR}$ spectra of the whole cells acquired as described in the methods. Cell viability was then checked microscopically and by reculturing in fresh growth medium. Cells were found to be 99% intact following data acquisition and all cultures

grew successfully. Plasma membranes were then isolated from the whole cells as described in the methods, suspended in β -buffer diluted twenty-fold with 95% deuterium oxide and the ^{19}F -NMR spectra again acquired. Membrane polar lipids were subsequently extracted, lyophilized and rehydrated in β -buffer (diluted twenty-fold with 95% deuterium oxide) by gentle heating and mild vortexing and once more the ^{19}F -NMR spectra were acquired. Finally, the thermotropic phase properties of the polar lipid fraction were determined via DSC and the polar lipid fatty acid composition analyzed by GLC.

Many fatty acids will, by themselves, support the growth of *A. laidlawii* B in the presence of an inhibitor of *de novo* fatty acid biosynthesis such as avidin - often leading to the production of membranes containing up to 99% of a single fatty acid species - but palmitic acid is not one of these (Silvius and McElhaney, 1978a). The fatty acid compositions of the membrane polar lipids from cells grown in the absence of avidin on media supplemented with 80 mole % palmitic acid plus 20 mole % of a particular MFPA are shown in Table 3. The fatty acid compositions were similar regardless of the particular positional isomer of MFPA present. In addition, the products of *de novo* fatty acid biosynthesis in *A. laidlawii* B (12:0, 14:0, 16:0 and 18:0) contributed significantly to the overall composition.

The percent enrichment with exogenously supplied fatty acids (the sum of palmitic acid plus MFPA) was between 75%

Table 3: Fatty Acid Composition of *A. laidlawii* Membrane Polar Lipids Isolated from Cells Supplemented With Palmitic Acid Plus Various Monofluoropalmitic Acids.

Fatty Acid Composition (Mole %)						
Supplement (0.12 mM Total)	12:0	14:0	16:0	MF16:0	18:0	
20% 6F16:0 + 80% 16:0	4.54	18.94	64.75	10.43	1.44	
20% 8F16:0 + 80% 16:0	0.91	16.51	69.81	12.46	0.31	
20% 10F16:0 + 80% 16:0	2.84	18.78	67.17	10.35	0.86	
20% 12F16:0 + 80% 16:0	2.97	16.82	72.23	7.82	0.16	
20% 14F16:0 + 30% 16:0	3.77	18.80	64.00	11.63	1.80	

Exogenous % Incorporation*	Ratio MF16:0/16:0
75.18	0.161
82.27	0.178
77.47	0.154
80.05	0.108
75.63	0.185

* Sum of mole percent 16:0 plus MF16:0

and 82% but this may be an overestimate due to the contribution of the *de novo* biosynthesis of palmitic acid. Hence, the fact that the ratio of MFPA to palmitate present in the membrane did not correspond to that provided in supplement was most probably not due to a disproportionate uptake of either of the supplementary fatty acids - MFPA's are proportionately incorporated in all cases tested to date (McDonough *et al.*, 1983; Macdonald *et al.*, 1983) - but rather may be attributed to the obscuring effect of *de novo* biosynthesis. Regardless, the levels of MFPA present were sufficient for the practical purpose of obtaining ^{19}F -NMR spectra.

Figure 12 shows the membrane polar lipid phase transition endotherms obtained using DSC for each case of coenrichment with a particular isomeric MFPA. The transition endotherms were typically somewhat asymmetric and similar to those of *A. laidlawii* B membranes and lipids previously reported from this laboratory (see, for example, McDonough *et al.*, 1983).

Excluding the thermotropic transition obtained in the case of coenrichment with 10F16:0, the midpoints of the phase transitions (T_m) varied over a range of approximately 1.0°C about the average T_m of 308.9°K . It is not clear why the particular case of 10F16:0 exhibited a T_m approximately 2.0°C lower than average. The phase transition temperatures reported here for enrichment with 80 mole % palmitic acid plus 20 mole % MFPA are somewhat lower than those which have

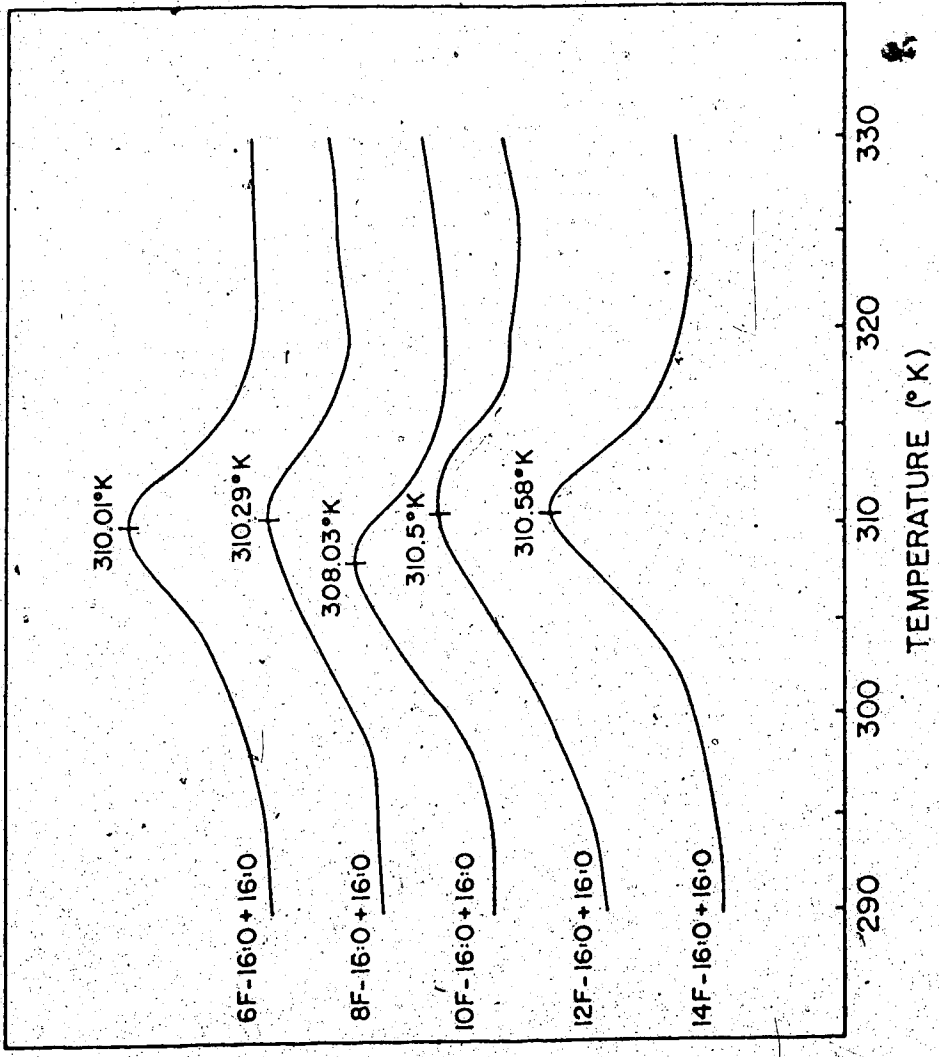


Figure 12: Main lipid phase transition endotherms obtained via DSC of isolated A. laidlawii B membrane polar lipids for each case of supplementation with 20 mole % xF16:0 plus 80 mole % 16:0.

previously been reported for enrichment of *A. laidlawii* B membranes with palmitic acid alone (De Kruffy *et al.*, 1973; McDonough *et al.*, 1983). However, levels of enrichment being somewhat variable and *de novo* biosynthesis making up any shortfall, leads to the situation apparent from Table 3, where myristic acid makes up nearly 20 mole % of the membrane fatty acids and, hence, lowers the T_m .

The parameter ΔT_{10-90} was of the order of 11°C to 12°C, in good agreement with those values previously reported from this laboratory (Silvius *et al.*, 1980).

The correspondence of experimentally acquired ^{19}F -NMR spectra and "best fit" model spectra is illustrated in Figure 13 for the case of cells supplemented with 20 mole % 8F16:0 plus 80 mole % 16:0. The spectra were acquired at temperatures at which the membrane lipids were just above (323°K), just below (300°K) and far below (279°K) their gel to liquid-crystalline phase transition. Whether obtained with whole cells, isolated membranes or membrane polar lipids, the ^{19}F -NMR spectra were remarkably similar, exhibiting identical changes with alterations in temperature and lipid phase state. At all temperatures and regardless of the lipid phase state, the ^{19}F -NMR spectra were adequately simulated using the model which assumed an axially symmetric line shape. The values of S_{m0} increased from approximately 0.20 to 0.82 while Δ_0 increased from about 100 Hz to 1600 Hz as the acquisition temperature was lowered from 323°K to 279°K.

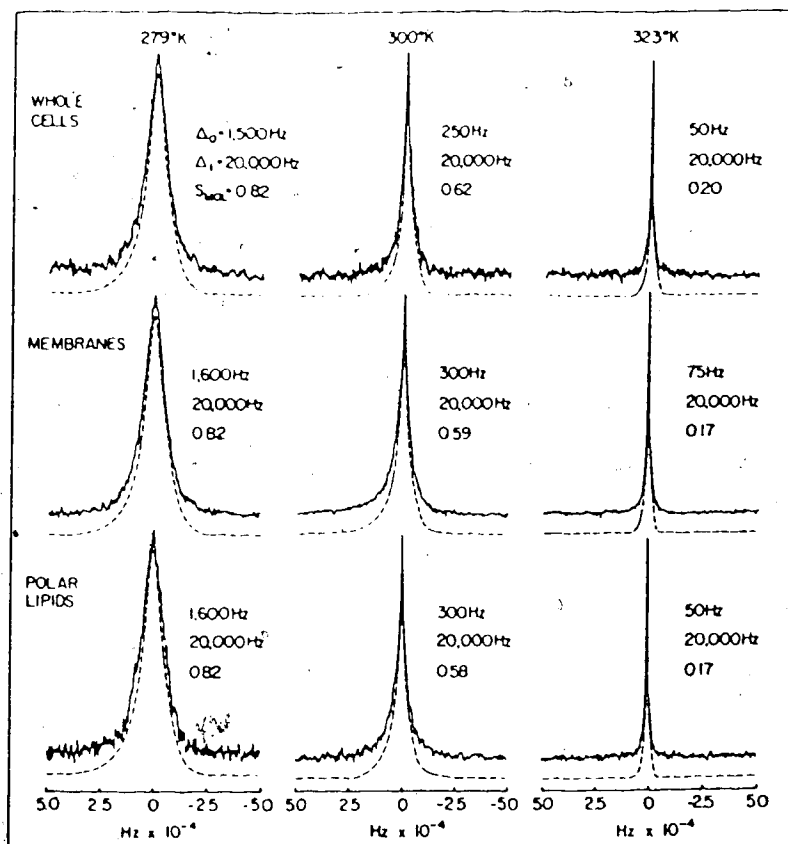


Figure 13: Correspondence of experimental ^{19}F -NMR spectra (whole cells, membranes and lipids of *A. laidlawii* enriched with 80 mole % 16:0 plus 20 mole % 8F16:0) and "best fit" simulated spectra (----). Experimental spectra were acquired with 100K, 50K and 25K acquisitions at 279°K, 300°K and 323°K, respectively. The corresponding values of the line shape parameters, Δ_0 , Δ_1 and S_{mol} used to generate the "best fit" simulated spectra are indicated.

The variation of S_{m01} with the position of the fluorine substituent provides an orientational order profile for a particular membrane. The ^{19}F -NMR order profiles of *A. laidlawii* B whole cells, isolated membranes and extracted polar lipids are shown in Figure 14. At 323°K, the values of the order parameters were relatively constant out to approximately carbon atom number ten and thereafter decreased as the nuclear spin probe was relocated further towards the methyl terminus of the acyl chain. Similar order profiles have been reported using ^2H -NMR techniques in both model (Seelig, 1977) and biological (Stockton *et al.*, 1977) membranes.

With decreasing temperature, orientational order generally increased. At 300°K, where the calorimetric data indicate that the phase transition is at least 90% complete, the average overall chain order was approximately 0.6, indicating that, despite the overwhelming preponderance of gel-state lipid, a substantial degree of disorder remained. The order profile itself indicated that, although a gradient of disorder was still present, the "incline" of the gradient was much less pronounced than that observed with entirely liquid-crystalline lipid at 323°K.

Upon decreasing the temperature further to 279°K, the average order parameter increased to approximately 0.8, indicative of a high degree of acyl chain ordering. In addition, all chain positions displayed an equal degree of ordering with the exception of the fourteen position.

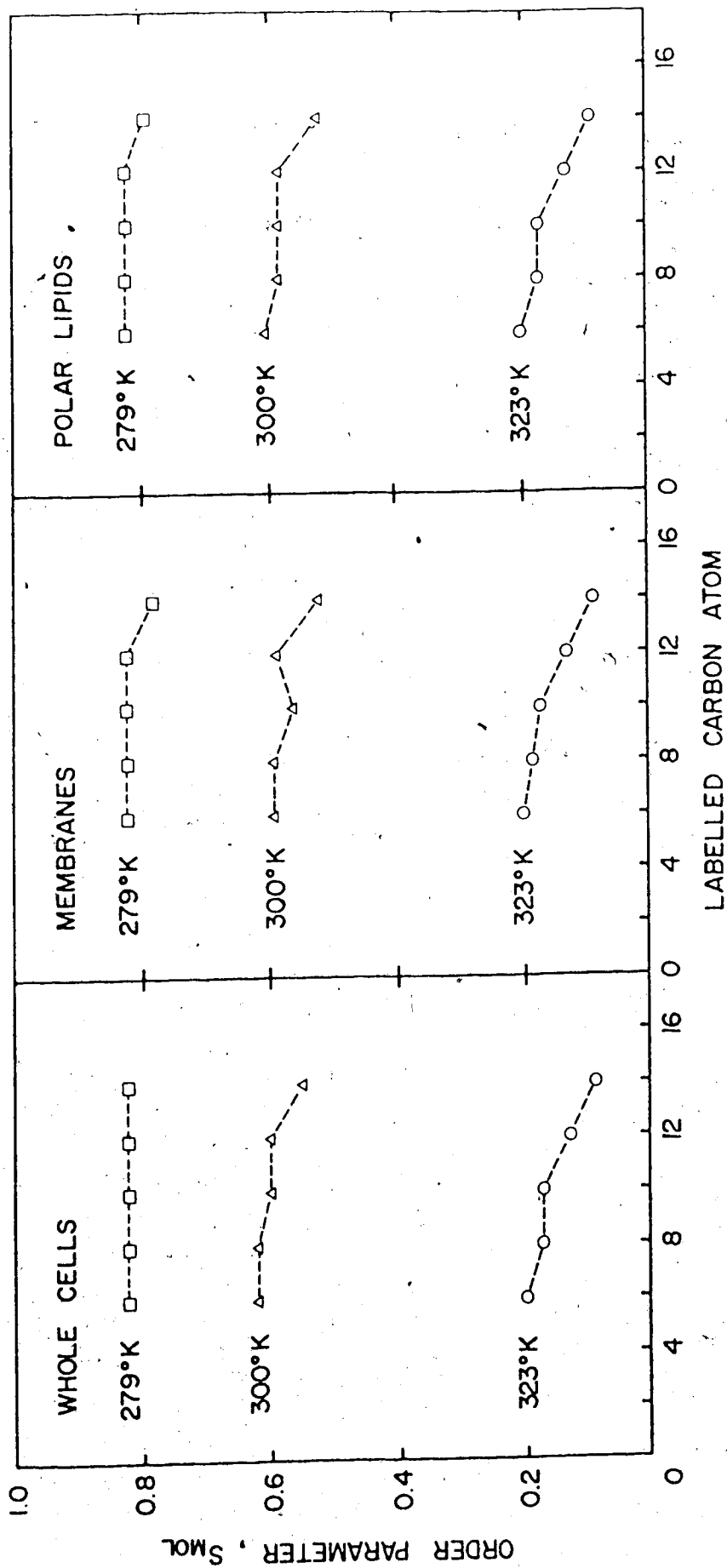


Figure 14: ¹⁹F-NMR orientational order profiles for whole cells, membranes and membrane polar lipids of *A. laidlawii* B enriched with palmitic acid.

Therefore, although some degree of disorder may exist near the methyl terminus, at temperatures well below the lipid phase transition the bulk of the acyl chain experiences an equally high degree of orientational order.

Allegrini *et al.* (1983) have studied the gel-state ^2H -NMR spectra of specifically deuterated palmitic acids mixed in equimolar amounts with 1-palmitoyllyso PC. These compounds together assume a bilayer structure, most probably via the formation of a functional dimer of lysophospholipid and free fatty acid. The free deuterated palmitic acid experiences a greater degree of rotational freedom in the gel-state which permits an analysis of the ^2H NMR spectrum in terms of orientational order parameters. The values of S_{m0} , obtained in the gel-state nearly approached the theoretical maximum of unity for positions C-2 to C-13 but thereafter decreased towards the acyl chain methyl terminus. These results substantiate our observations of a highly ordered state with a minimal number of *gauche* conformers in the gel phase. However, the maximum value of S_{m0} reported by Allegrini *et al.*, (1983) was 0.94, which is some 15% higher than the maximum of 0.82 which we have obtained. Although it is possible to question the applicability of results obtained with a mixture of lysophospholipid and free fatty acid to the situation in a diacyl phospholipid membrane, this difference in maximum ordering is most probably attributable to the considerable fatty acyl chain heterogeneity of the *A. laidlawii* B membrane lipids in

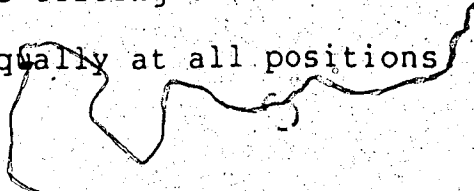
contrast to the absolute homogeneity of the model membrane system. It is possible to demonstrate, for example, that the presence of shorter carbon chains markedly reduces orientational order in lyotropic nematic phases (Covello *et al.*, 1983).

It would be premature, however, to exclude the possibility that lipid headgroup heterogeneity makes some additional contribution to gel-state disorder in *A. laidlawii* B. As discussed by Seelig and Browning (1978), when the ^2H -NMR order parameters of acyl chains esterified to a variety of lipid headgroup classes are compared in the liquid-crystalline state at a temperature normalized relative to their respective phase transitions, the values are all very similar (but see Marsh *et al.*, 1983). Again in the liquid-crystalline state, Rance *et al.* (1983), in a ^2H -NMR study, could discern little dependence of conformational order on headgroup class amongst *A. laidlawii* B glycerolipids. Such generalizations may not apply in the gel-state, where the densities of acyl chain and headgroup packing become more pronounced and interdependent. Indeed, without even considering the respective gel-state stabilities of the individual classes of glycerolipids present in *A. laidlawii* B, their heterogeneity alone could constitute a barrier to complete orientational ordering.

The 60 KHz splitting which appears in the gel-state ^2H -NMR spectrum of *A. laidlawii* B enriched with deuterated fatty acids has been interpreted as indicating that the acyl

chains still retain considerable motional freedom at low temperatures (Smith *et al.*, 1979). These authors concluded that at temperatures just below the lipid phase transition, the acyl chains have assumed an all-*trans* configuration and that only "rotational disorder" resulting from rapid rotation about the long molecular axis, and not *trans-gauche* isomerizations, reduce the observed quadrupolar splitting from the theoretical maximum of 126 KHz to the observed 60 KHz. The interpretation has been challenged by Pink and Zuckermann (1980), who point out that the results of Raman scattering studies indicate that there are a non-negligible number of *gauche* bonds excited in the gel-state of pure PC's. From consideration of a theoretical model of a biological membrane, these authors concluded that the gel-state $^2\text{H-NMR}$ spectra in *A. laidlawii* B could be interpreted as indicating the occurrence of *trans-gauche* isomerizations in addition to rotational disorder in the gel-state.

NMR-derived orientational order parameters are modulated by whole chain tilt as well as by *trans-gauche* isomerizations at individual chain segments (Petersen and Chan, 1977). A straight-chain, saturated fatty acid in the all-*trans* configuration, experiencing only rotational disorder and/or fluctuations in the orientation of the long molecular axis, should not manifest a gradient of disorder along its length, since tilting of the entire acyl chain should decrease S_{m0} equally at all positions. The $^{19}\text{F-NMR}$



order profiles observed at 300°K indicate that at temperatures just below the lipid phase transition a gradient of disorder is still present, implying that the acyl chain has not assumed an all-*trans*-configuration regardless of the contribution of whole chain fluctuations to the average order parameter. While the observation of an order gradient precludes the existence of an all-*trans* configuration, the converse, that the absence of an order gradient implies the all-*trans* state, does not hold. A population of *gauche* isomers distributed with equal probability or diffusing rapidly along the acyl chain could diminish S_{m0} , homogeneously, as observed at 279°K in *A. laidlawii* B via ^{19}F -NMR.

It is interesting to note that few if any differences in orientational order can be discerned between live cells, membranes and polar lipids. Studies of orientational order in isolated biological membranes would, therefore, appear to be directly applicable to the situation in whole cells. It is equally significant that the absence of integral membrane proteins had no effect upon S_{m0} . While it has been clear for some time that values of S_{m0} obtained by ^2H -NMR techniques are nearly identical whether obtained in model or biological membranes in the liquid-crystalline state (Seelig and Seelig, 1980), this point had not been demonstrated for ^{19}F -NMR techniques.

Jarrell *et al.* (1982) investigated the ^2H -NMR spectra of *A. laidlawii* B whole cells, membranes and lipids enriched

with deuterated myristic acid, while Kang *et al.* (1981) compared $^1\text{H-NMR}$ splittings in *A. laidlawii* B membranes and extracted lipids enriched with deuterated myristic acids. These authors were able to discern little difference in the quadrupolar splittings obtained in cells, membranes or lipids. These results are in agreement with our conclusions concerning the applicability of studies in isolated lipids and membranes to the membrane lipid organization in intact cells. Furthermore, both $^1\text{H-NMR}$ and $^{19}\text{F-NMR}$ results agree that the presence of membrane proteins does little to alter the range of conformations available to membrane fatty acyl chains in the liquid-crystalline state. This apparent lack of influence on lipid order by membrane proteins may be the result of the relatively low protein/lipid ratio in *A. laidlawii* B compared to the ratio in model systems where some effects are discernible (Kang *et al.*, 1981). The lateral segregation of intrinsic membrane proteins into protein rich domains, which can be observed by electron microscopy in *A. laidlawii* B membranes cooled below the lipid phase transition temperature (Silvius and McElhaney, 1980), further suggests that, in the gel-state, bulk membrane lipid conformations should not be, and as concluded from $^{19}\text{F-NMR}$ data, are in fact not particularly influenced by membrane proteins.

An Isomeric Series of *CIS*-Octadecenoic Acids

The *CIS*-double bond occurs widely in biological membranes and has been the subject of recent $^1\text{H-NMR}$ studies in both model (Seelig and Seelig, 1977; Seelig and Waespe-Sarčević, 1978) and biological (Rance *et al.*, 1980) membranes. These studies indicated that conformational order was reduced in the presence of *CIS*-unsaturated acyl chains relative to saturated acyl chains when measured at the same temperature. When compared at the same temperature relative to their respective lipid phase transition points, the *CIS*-unsaturated acyl chains displayed nearly identical or a somewhat greater degree of orientational order than straight-chain saturated acyl chains, suggesting that the conformations available to saturated and unsaturated acyl chains in the liquid-crystalline state were nearly identical. It was concluded that a local "organizational perturbation" induced by the *CIS*-double bond determines the proportion of gel and liquid-crystalline lipid. The nature of this perturbation remains ill defined. As pointed out by Barton and Gunstone (1975), simple gel-state chain packing considerations seem insufficient to explain the relationship between the temperature of chain melting and the position of a *CIS*-double bond along an acyl chain.

The availability of a series of positional isomers of *CIS*-octadecenoic acid (Gunstone and Ismail, 1967a), coupled with the ability to rapidly screen a range of fatty acid structures via $^{19}\text{F-NMR}$, led me to undertake a systematic

survey of the relationship between lipid orientational order and the position of a *CIS*-double bond in membranes of the organism *A. laidlawii* B.

The ^{19}F -NMR spectra of membranes of *A. laidlawii* B enriched with small amounts of one of a series of positional isomers of monofluoropalmitic acid, plus one of a series of positional isomers of *CIS*-octadecenoic acid ranging from $\Delta 4$ through $\Delta 15$ inclusive, were acquired at a variety of temperatures and analyzed in terms of conformational order parameters. It was demonstrated that while orientational order was very similar in the liquid-crystalline state regardless of double bond position, significant differences in chain ordering were manifest in the gel-state with different sites of unsaturation. These results suggest that, even in the densely packed gel-state, regions of the fatty acyl chains differ in their susceptibilities to conformational perturbation. Nevertheless, normalization of the S_{m0} values with respect to the gel to liquid-crystalline phase transition indicated that the lipid phase state remains the preeminent determinant of overall orientation order.

The fatty acid compositions of membranes of *A. laidlawii* B, enriched with each of the isomeric *CIS*-octadecenoic acids studied, are listed in Table 4 for the typical case of coenrichment with 8F16:0. When other monofluoropalmitic acids were substituted, similar results were obtained. In all cases the products of *de novo* fatty

Table 4: Fatty Acid Composition of Membranes of *Acholeplasma laidlawii* B Strains with 8F16:0 plus a particular isomer of *cis*-octadecenoic acid.

Fatty Acid Supplement	Membrane Fatty Acid Composition (MOLE %)									
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	18:4	18:5
15% 8F16:0 + 85% 18:1cΔ4	1.0	1.0	0.8	-	-	85.1	-	-	-	11.3
15% 8F16:0 + 85% 18:1cΔ5	-	1.4	3.1	-	-	85.7	-	-	-	9.8
15% 8F16:0 + 85% 18:1cΔ6	-	0.9	0.7	0.3	-	83.1	-	-	-	14.9
20% 8F16:0 + 80% 18:1cΔ7	0.1	1.4	1.0	6.8	0.1	73.4	-	-	-	17.2
20% 8F16:0 + 80% 18:1cΔ8	1.2	0.6	1.8	6.0	-	72.4	-	-	-	18.0
20% 8F16:0 + 80% 18:1cΔ9	0.9	1.2	2.1	8.1	-	72.1	-	-	-	15.6
20% 8F16:0 + 80% 18:1cΔ10	0.3	1.0	4.2	5.8	1.0	69.8	-	-	-	17.9
15% 8F16:0 + 85% 18:1cΔ11	-	5.4	9.2	-	-	64.2	-	-	-	21.2
20% 8F16:0 + 80% 18:1cΔ12	1.0	0.9	1.9	5.9	-	72.3	-	-	-	18.0
10% 8F16:0 + 90% 18:1cΔ13	1.8	1.5	0.8	2.3	1.0	81.2	-	-	-	11.3
15% 8F16:0 + 85% 18:1cΔ14	1.6	1.0	0.8	-	-	84.2	-	-	-	12.3
15% 8F16:0 + 85% 18:1cΔ15	0.9	0.8	1.3	-	-	84.0	-	-	-	12.0

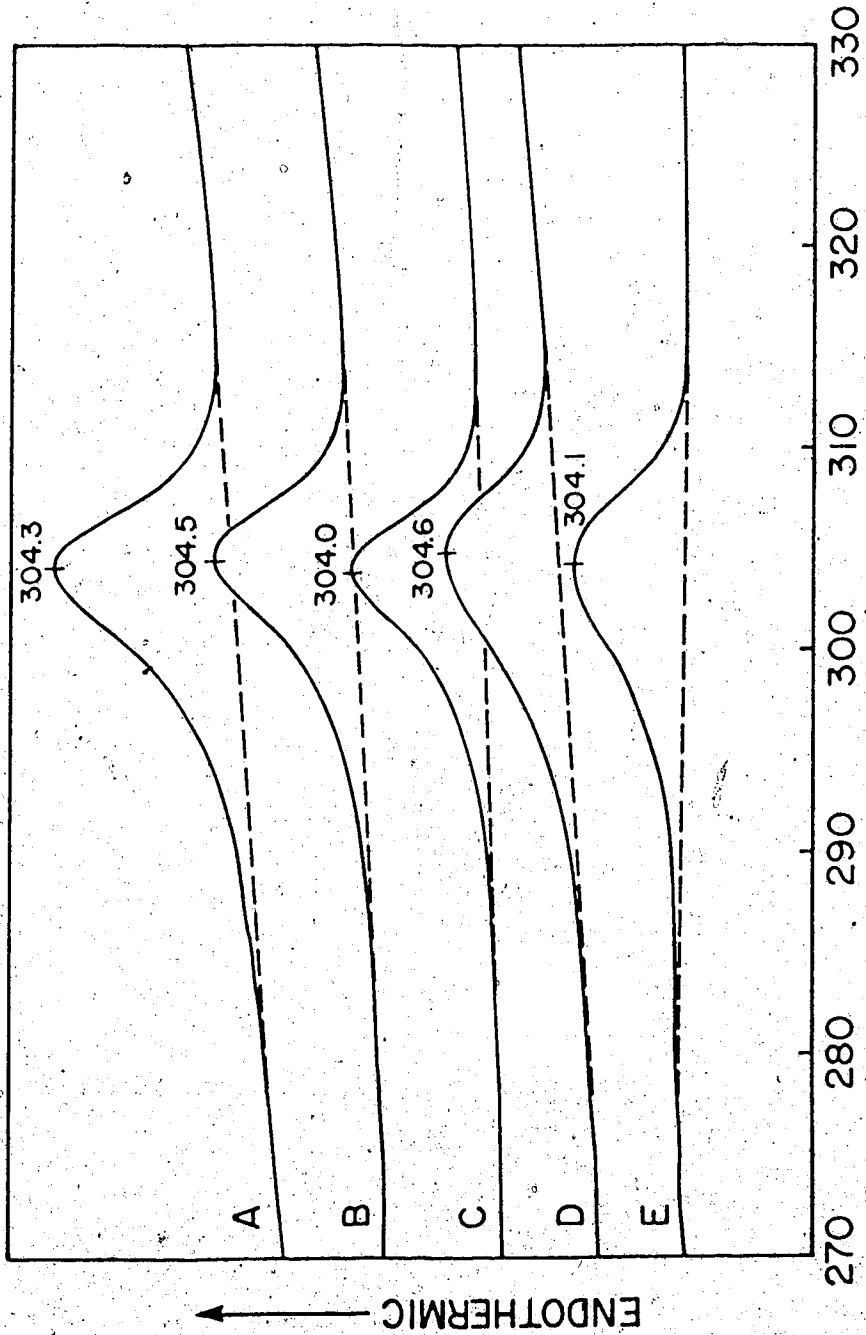
acid biosynthesis in *A. laidlawii* B, specifically 12:0, 14:0, 16:0 and 18:0 (Saito *et al.*, 1977), contributed minimally to the membrane fatty acid composition, reflecting the effects of inhibition with avidin (Silvius and McElhaney, 1978a). In turn, the combination of fatty acids provided exogenously together generally accounted for more than 90% of the total membrane fatty acids present. Levels of enrichment were greatest when the physical properties of the particular *cis*-octadecenoic acid presented the least challenge to the organism's ability to grow (Silvius and McElhaney, 1978a). The membranes enriched with 18:1 Δ 7, Δ 8, Δ 9, Δ 10 or Δ 12 contained significant quantities of 16:1 in addition to the fatty acids provided in supplement. Since *A. laidlawii* B can neither biosynthesize nor degrade

unsaturated fatty acids, it must be assumed that this "aminant" was concentrated from background levels in the (Saito *et al.*, 1977).

The ratio 18:1 Δ _x/x Δ 16:0 present in the membrane lipids generally corresponded to that originally provided exogenously, indicating that, as demonstrated previously (McDonough *et al.*, 1983; Macdonald *et al.*, 1983), the monofluoropalmitic acids are readily incorporated by *A. laidlawii* B. The generally high levels of supplement incorporation and low levels of background *de novo* biosynthesis would indicate that the reported order parameters represent almost exclusively the effect of the *cis*-octadecenoic acid of interest upon the orientational

order of the monofluoropalmitic acid probe.

The position of the phase transition of the polar lipids extracted from each membrane sample employed were determined utilizing DSC. Representative endotherms are illustrated in Figure 15. The endotherms were typically somewhat asymmetric and similar to transition endotherms of *A. laidlawii* B membrane lipids reported previously from this laboratory. For any particular isomer of *cis*-octadecenoic acid, the T_m of the membrane polar lipids varied only 1° to 2°C over the range of monofluoropalmitic acids utilized. Although transition enthalpies were not calculated in each case, where estimated, ΔH was approximately 80% of that of the corresponding *di*-octadecenoyl PC (Barton and Gunstone, 1975). Values of ΔT_{10-90} were in these cases, in the range of 6° to 11°C, in good agreement with values reported previously from this laboratory for *A. laidlawii* B membrane lipids highly enriched with a variety of fatty acids (Silvius *et al.*, 1980). Figure 16 illustrates the variation of T_m with the position of the *cis*-double bond along the eighteen-carbon chain. The T_m 's of the corresponding synthetic *di-cis*-octadecenoyl PC's (Barton and Gunstone, 1975) are included for comparative purposes. In both cases the T_m 's were minimal when the *cis*-double bond was located near the center of the acyl chain and increased progressively as the double bond was relocated towards either the carbonyl headgroup or the methyl terminus of the acyl chain. The alternation of the melting points exhibited



TEMPERATURE (°K)

Figure 15: The gel to liquid-crystalline phase transition endotherms of total membrane polar lipids extracted from membranes of A. laidlawii B enriched with 15% MFPA plus 85% 18:1cΔ4. A, 6F16:0; B, 8F16:0; C, 10F16:0; D, 12F16:0; E, 14F16:0. The scan rate was 5°C/min. The dashed line represents the interpolated baseline.

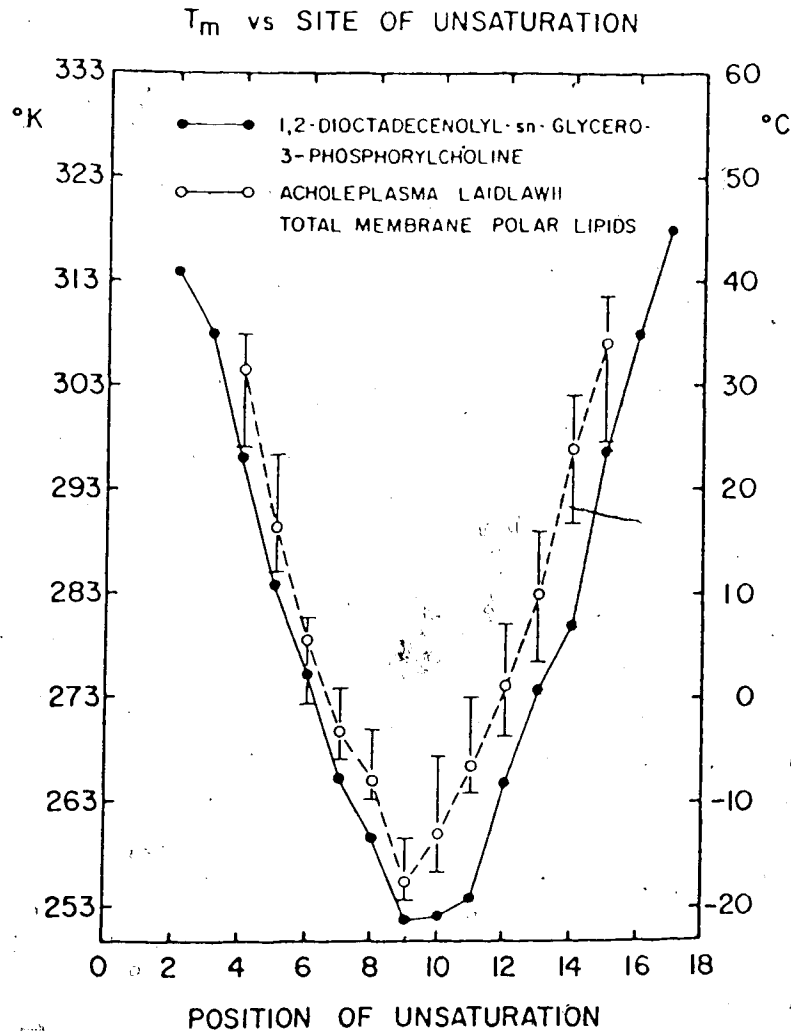


Figure 16: Variation of T_m with the position of the site of cis-unsaturation in A. laidlawii B total membrane polar lipids. The data on di-cis-octadecenoyl-phosphatidyl cholines was taken from Barton, P.B. and Gunstone, F.D. (1975) J. Biol. Chem. 250, 4470-4476. Open circles, A. laidlawii B total membrane polar lipids; closed circles, 1,2-dioctadecenoyl-sn-glycero-3-phosphorylcholines. The brackets represent the values of ΔT_{10-90} .

by the fatty acids themselves (Gunstone and Ismail, 1967b) was not observed. Values of T_m for the *A. laidlawii* B membrane polar lipids were consistently greater than those of the corresponding *di-cis*-octadecenoyl PC's. This effect may be attributed to the presence in the *A. laidlawii* B membrane lipids of 10% to 20% of the generally higher-melting point monofluoropalmitic acid as well as to differences arising solely as a result of the glycerolipid headgroup composition of *A. laidlawii* B membrane lipids.

^{19}F -NMR order profiles were acquired at a variety of temperatures for each isomer of *cis*-octadecenoic acid from $\Delta 4$ through $\Delta 15$ inclusive. These order profiles are illustrated in Figure 17. At 310°K all isomers exhibited the progressive decrease of orientational order, with its decline towards the methyl termini of the acyl chains, which is characteristic of the order profiles obtained by other techniques, such as ^2H -NMR, at temperatures above the gel to liquid-crystalline phase transition (Seelig, 1977; Stockton *et al.*, 1977). Provided that over the range of temperatures studied the membrane lipids remained in the liquid-crystalline state, the shape of the order profiles remained relatively constant with decreasing temperature, while overall orientational order increased marginally. This situation can be seen to apply particularly to the cases of the $\Delta 9$ and $\Delta 11$ isomers and similar results have been obtained previously using both ^2H -NMR (Seelig and Seelig, 1977) and ^{19}F -NMR (Macdonald *et al.*, 1983) techniques. In

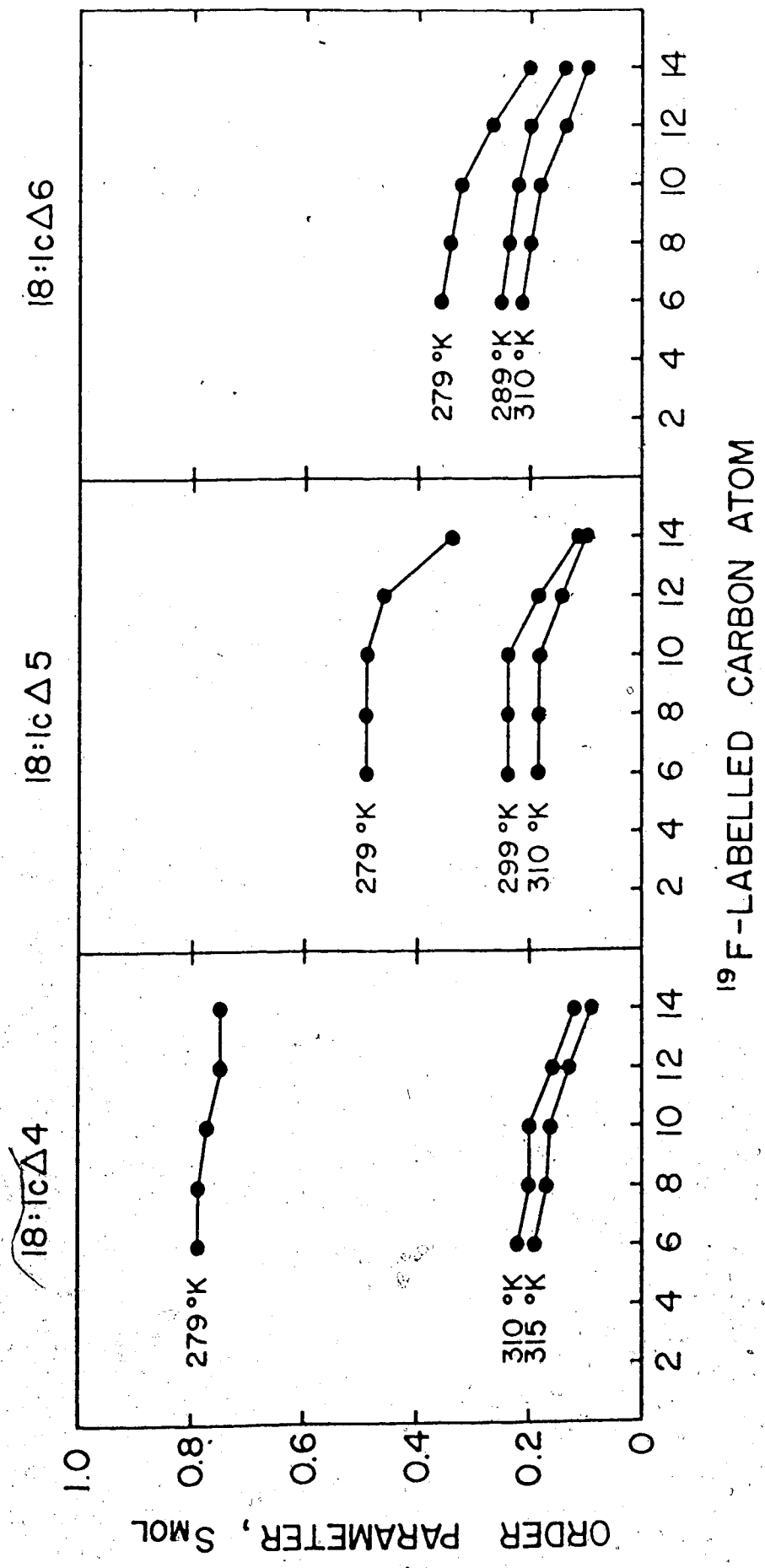


Figure 17: ^{19}F -NMR orientational order profiles of membranes of A. laudlawii B enriched with each of an isomeric series of cis-octadecenoic acids.

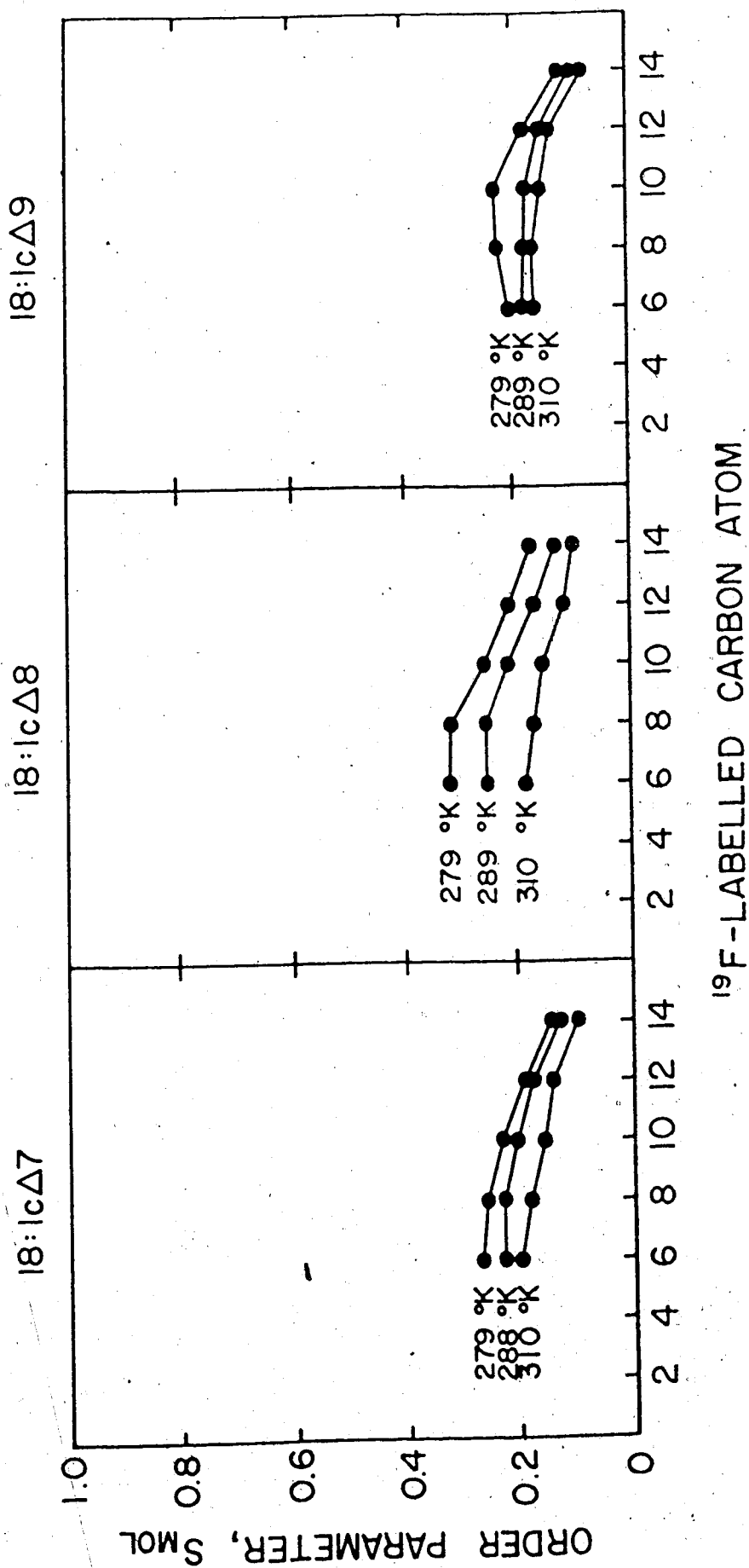
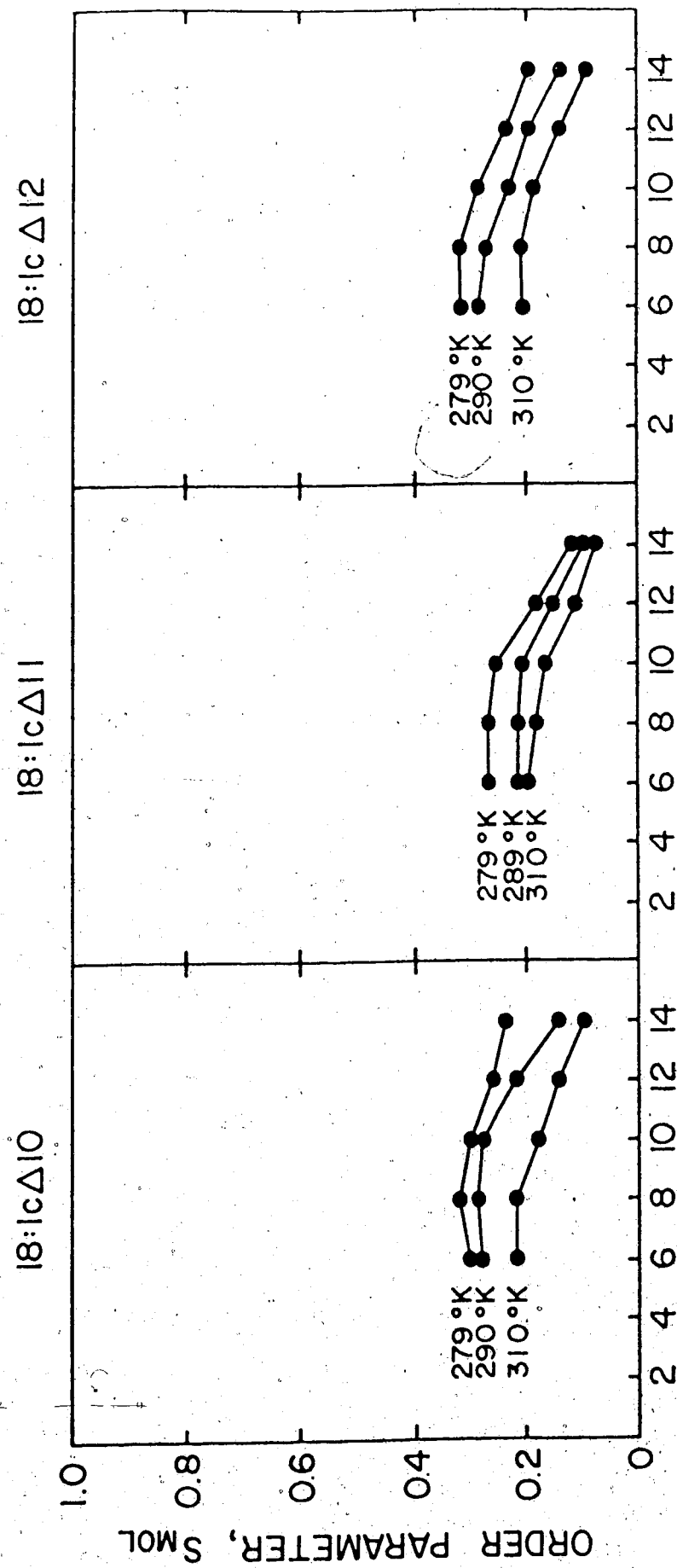
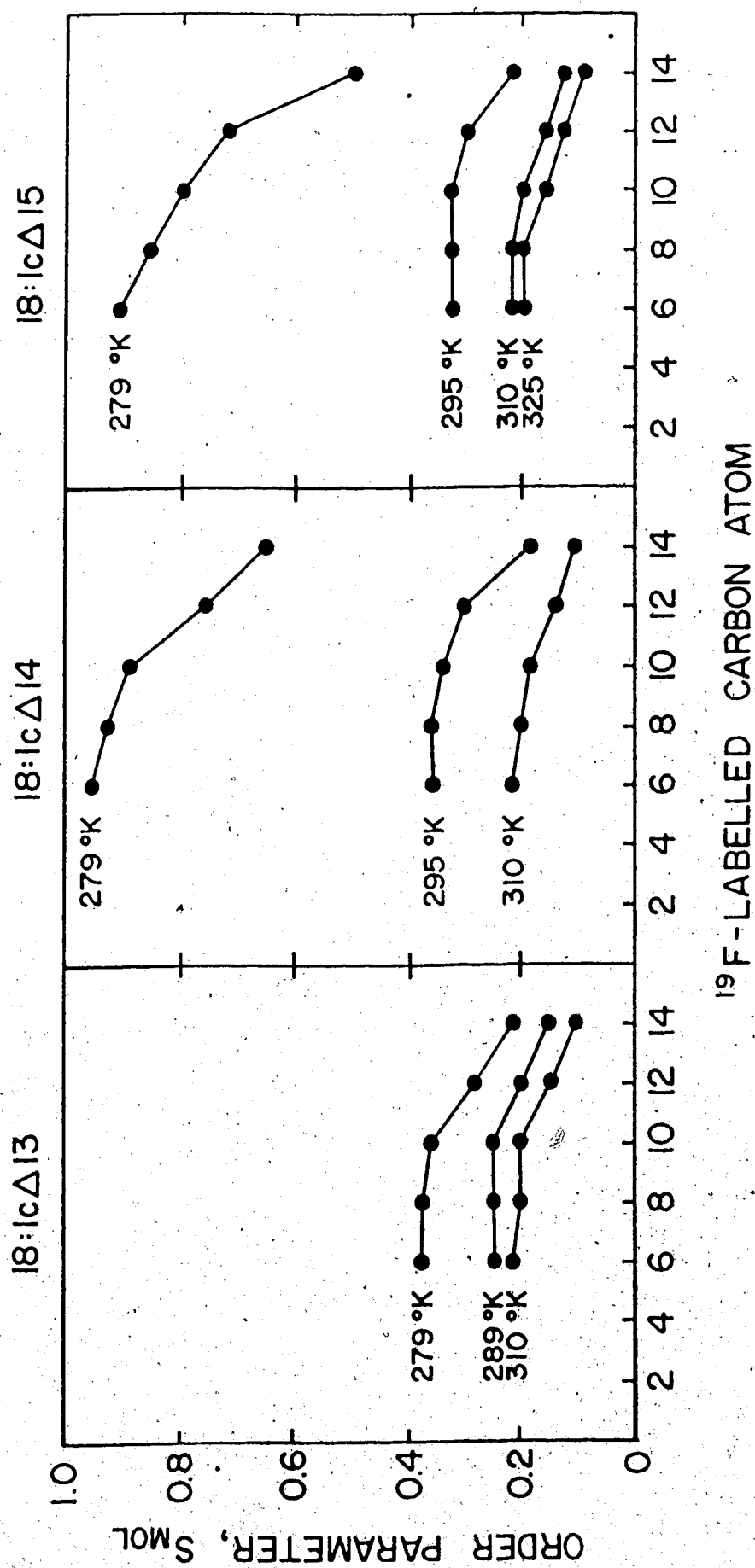


Figure 17 continued.



^{19}F -LABELLED CARBON ATOM

Figure 17 continued.



^{19}F -LABELLED CARBON ATOM

Figure 17 continued.

that the $^1\text{H-NMR}$ order profiles obtained at 310°K - where in each case the membrane lipids would have assumed the liquid-crystalline state - were highly similar regardless of the position of the site of unsaturation, there was no evidence of the local ordering effect of a *cis*-double bond upon a neighbouring saturated acyl chain as reported by Seelig and Seelig (1977). However, since we report order parameters only for every second methylene segment, this finding is not particularly surprising. When the temperature range which was employed overlapped into or entirely encompassed the gel to liquid-crystalline phase transition, there was a dramatic increase in overall orientational order with decreasing temperature and the order profiles for different isomers of *cis*-octadecenoic acid began to acquire distinct dissimilarities. At 279°K , the lowest temperature employed, only four of the *cis*-octadecenoic acid-enriched membranes contained large quantities of gel-state lipid and these corresponded to isomers with positions of unsaturations nearest either the carbonyl headgroup or the methyl terminus of the acyl chain.

The order profile, in the gel-state, of membranes enriched with $18:\text{c}\Delta 4$ was almost flat with individual values of S_{m^2} approaching 0.8, indicating a highly ordered state with all methylene segments experiencing an approximately equal degree of disorder. In contrast, the order profile of membranes enriched with $18:\text{1c}\Delta 15$, when obtained at 279°K , indicated that a gradient of disorder still existed with

S_{m01} varying from 0.86 nearest the carboxyl headgroup to approximately 0.5 near the fatty acyl chain methyl terminus. A similar gradient of disorder remained at 279°K in membranes enriched with 18:1c Δ 14. In addition, a comparison of the order profile at 279°K in the case of enrichment with 18:1c Δ 5, with the order the profile obtained at 295°K in the case of enrichment with 18:1c Δ 15, again tends to indicate that the disorder gradient is much more pronounced in the gel-state when the site of unsaturation is near the methyl terminus of the acyl chain than when it is near the carboxyl headgroup, since at these temperatures these two isomers are in approximately equal physical states with respect to the gel to liquid-crystalline phase transition.

The effects of *cis*-unsaturation on molecular order in the liquid-crystalline state have been studied in both model and biological membranes. Seelig and Seelig (1977) compared the ordering of the palmitic acyl chain of POPC with its equivalent in DPPC via ¹H-NMR. Although a local ordering effect of the *cis*-double bond could be discerned in the order profile of the neighbouring palmitic acyl chain, the head-to-tail distribution of order parameters was generally similar in the presence or absence of an adjacent *cis*-double bond. When compared at the same temperature, the palmitic acyl chains in DPPC were overall more ordered than the palmitic acyl chain in POPC. This result could be directly related to the lower lipid phase transition temperature of POPC (-5°C) relative to DPPC (41.8°C). However, when

compared at a constant temperature relative to their respective phase transition temperatures, the palmitic acyl chains of DPPC were overall *less ordered* than those in POPC. Thus the *cis*-double bond restricted the range of conformations available to the adjacent acyl chain and this characteristic was evident only when the two systems were compared under conditions where they were subjected to the same average molecular forces.

The quadrupolar splittings of specifically deuterated *sn*-2 oleic acyl chains in POPC exhibited a sharp minimum at the C-10 position, the location of the *cis*-double bond (Seelig and Waespe-Sarčević, 1978). This observation could be explained in terms of an average inclination of the *cis*-double bond of 7-8° with respect to the bilayer normal. When corrected for this geometric consideration, the order parameter profiles of the unsaturated *sn*-2 acyl chain were very similar to those of the saturated *sn*-1 chain or POPC.

Gally *et al.* (1979) incorporated specifically deuterated oleic acids into the membrane lipids of a fatty acid auxotroph of *E. coli* and observed the same sharp minimum in the positional dependence of the quadrupolar splittings at the C-10 position that was observed in the model POPC system. Rance *et al.* (1980) demonstrated that the orientation of the rigid *cis*-double bond inferred from the quadrupolar splittings in POPC and *E. coli* was manifest as well in membranes of *A. laidlawii* B enriched with specifically deuterated oleic acids.

The ^2H -NMR studies agree then that in the liquid-crystalline state, in both model and biological membranes, the shape of the order profile is relatively constant, whether it be the oleic acyl chain after correction for geometric considerations or a neighbouring palmitic acyl chain. Moreover, specifically deuterated elaidic acyl chains incorporated into PEPC exhibit an order profile very similar to POPC or DPPC (Seelig and Waespe-Sarčević, 1978). Although it is possible to discern the effects of specific structural substituents upon the shape of the order profile in the liquid-crystalline state via ^{19}F -NMR (Macdonald *et al.*, 1983) as well as by ^2H -NMR (Seelig and Seelig, 1977), these changes are small when compared to the temperature dependence of the order parameters. It seems likely that the plasticity of the liquid-crystalline state is sufficient to accommodate a diversity of fatty acyl structural substituents without seriously affecting the head-to-tail gradient of configurational probabilities. The present observation that, provided the *A. laidlawii* B membrane lipids were in a liquid-crystalline state, the ^{19}F -NMR order profiles were similar regardless of the particular isomeric *cis*-octadecenoic acid present, lends further credence to this conclusion.

Further to this point, it is instructive to note that the positional dependence of the quadrupolar splittings obtained with specifically deuterated fatty acids incorporated into nematic, smectic or cholesteric

liquid-crystal hosts display a head-to-tail gradient of orientational order similar to that observed in a lipid bilayer (Forest and Reeves, 1979; Covello *et al.*, 1983; Davis, 1983; Alcantara *et al.*, 1983). Here the common denominator is a preferred orientation of the molecules along a particular axis, and chain motions which would alter the orientation of the entire acyl chain or a portion thereof are necessarily restricted to those which are concerted or cooperative. An acyl chain experiencing overall isotropic reorientations manifests a gradient of multiple internal rotations (e.g., *trans-gauche* isomerizations) which accumulate in frequency in a linear fashion from the centre of mass (the polar headgroup) towards the methyl terminus (Brown *et al.*, 1979). It is the interplay of these two forces in the lipid bilayer, restriction due to preferred orientation vs progressively accumulating internal reorientations, which is manifest in the observed bilayer orientational order profile. In the liquid-crystalline state, *trans-gauche* isomerizations and tilting of the acyl chains are (relative to the gel-state) highly probable and chain-packing restrictions much less severe. Thus it is not surprising that acyl chains in the liquid-crystalline state are able to accommodate structural substituents in any portion of the chain without markedly affecting the head-to-tail range of available conformations when their motional freedom is so great to begin with.

In the gel-state, fatty acyl chain-packing densities increase substantially and the restriction of acyl chain motional freedom is severe. Thus the orientational order profiles of straight-chain saturated fatty acids approach the theoretical maximum in the gel-state whether measured via $^2\text{H-NMR}$ (Allegrini *et al.*, 1983) or $^{19}\text{F-NMR}$. One might predict that the inclusion of structural substituents which perturbed the acyl chain packing and relieved the restriction of motional freedom would manifest this effect in the orientational order profile. The gel-state order profiles of membranes enriched with 18:1 Δ 14 or 18:1 Δ 15 indicate that when a *cis*-double bond is located proximal to the methyl terminus of an acyl chain, it perturbs the chain packing in the gel-state sufficiently to permit a substantial increase in motional freedom in its immediate vicinity. This can be most readily appreciated when it is noted that the $^{19}\text{F-NMR}$ gel-state order profiles of *A. laidlawii* B membranes enriched with palmitic acid are characteristically flat with any residual disordering being evenly distributed along almost the entire length of the acyl chain. When the *cis*-double bond is located near the acyl chain carbonyl headgroup, there is little evidence of a local increase in motional freedom but rather there is a decrease in orientational order at all chain segments relative to the comparable situation in palmitic acid-enriched *A. laidlawii* B membranes. This observation can be most readily interpreted if it is assumed that the

portion of the acyl chain posterior to the *cis*-double bond is tilted with respect to the bilayer normal and experiences an approximately equal and small degree of disordering at each chain position due to *trans-gauche* isomerization. This circumstance further suggests that the acyl chains are less susceptible to packing perturbations in the region near the carbonyl headgroup than near the methyl terminus. Therefore, even in the gel-state, the stringency of packing restrictions is subject to variation across the width of the bilayer. This conclusion is supported by a $^1\text{H-NMR}$ study of a mixture of deuterated palmitic acid and lysophospholipid (Allegrini *et al.*, 1983) in which it was reported that, although quadrupole splittings approached the theoretical maximum in the gel-state for chain segments C-2 to C-13, at positions further towards the chain terminus orientational order still decreased progressively and substantially.

Figure 18 compares the chain-average order parameters for each isomer of *cis*-octadecenoic acid at the reduced temperature, T_r . This comparison is intended to normalize the data with respect to the gel to liquid-crystalline phase transition so that effects attributable to differences in the phase transition temperature can be eliminated or at least minimized (Seelig and Seelig, 1980). The chain-average order parameters in Figure 18 all fall within a relatively narrow band, indicating that all isomers assume approximately similar states of orientational order at equal values of reduced temperature, regardless of the position of the

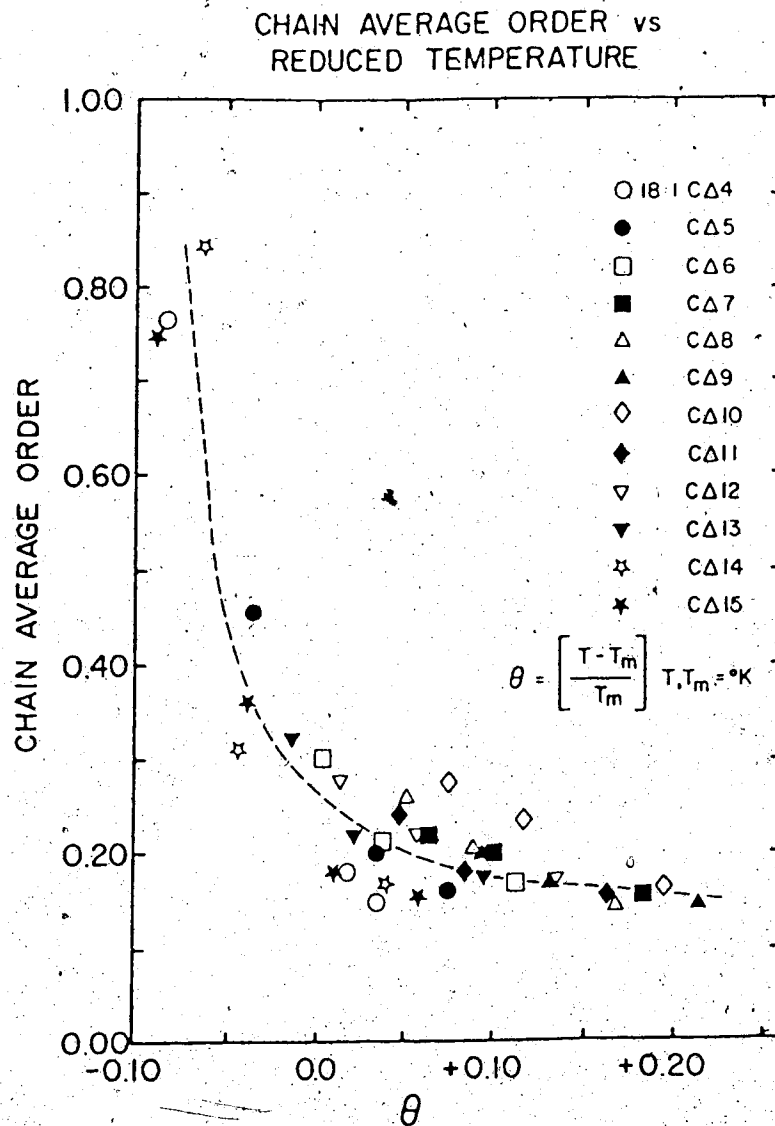


Figure 18: Normalization of the chain average order in the presence of isomeric cis-octadecenoic acids with respect to the lipid phase transition T_m . T_r and the chain average order were calculated as described in the text.

site of unsaturation. The average order increased slowly as the temperature decreased toward the lipid phase transition ($T_r = 0$), began to rise exponentially as that transition was traversed and plateaued at $S_{m0} \cong 0.8$. Although a direct comparison of the average order of all isomers at all values of reduced temperature is not possible with these data, it can be discerned that, in the region $0 < T_r < + 0.12$, isomers with double bonds situated near the centre of the fatty acyl chain are generally more ordered than those isomers with sites of unsaturation further towards either the carboxyl headgroup or the methyl terminus of the fatty acyl chain. In addition, the differences noted earlier in the order profiles of $\Delta 4$, $\Delta 5$, $\Delta 14$ and $\Delta 15$ isomers are manifest, particularly at $T_r = -0.04$.

Isomeric *trans*-Octadecenoic Acids

The *trans*-double bond, although not a common or usually even a natural constituent of biological membranes, has nevertheless been of interest in the experimental manipulation of membrane fatty acid composition. Fatty acids containing sites of *trans*-unsaturation are capable of supporting the growth of unsaturated fatty acid auxotrophs of *E. coli*, can be incorporated by *Mycoplasma capricolum* and *A. laidlawii* B to the extent that their membrane lipids are virtually homogeneous with respect to these fatty acids, and apparently are able to support normal membrane functioning in these circumstances (the effects of membrane lipids on

growth and membrane activities has recently been reviewed, by McElhaney, 1982a). The *trans*-double bond provides an intermediate level of fluidity between saturated and *cis*-unsaturated fatty acids. (for a recent review, see McElhaney, 1982b).

In this section I extend studies of the orientational order of MFPA's incorporated into the membrane lipids of *A. laidlawii* B in the presence of fatty acids containing various structural and positional substituents to the cases of three positional isomers of *trans*-octadecenoic acid.

The ^{19}F -NMR orientational order parameters of *A. laidlawii* B membranes enriched with small amounts of one of a series of positional isomers of MFPA plus large amounts of one of the *trans*-octadecenoic acids 18:1 Δ 6, Δ 9 or Δ 11, were obtained at temperatures corresponding to either the liquid-crystalline or the gel-state. The resulting bilayer order profiles provided evidence of specific local effects of the *trans*-double bond which could be correlated with the position of the site of unsaturation in either of the two phase states. Normalization of the orientational order data with respect to the calorimetrically determined gel to liquid-crystalline phase transitions then permitted a comparison of the overall temperature dependence of orientational order in the presence of *trans*-unsaturation with that observed in the presence of other structural substituents. A coherent picture of the manner in which the *trans*-unsaturated species affects the bilayer stability

emerged when these specific local and general overall effects were considered together.

Table 5 lists the fatty acid composition for each case of supplementation with a particular combination of isomeric *trans*-octadecenoic acid plus isomeric monofluoropalmitic acid. The membrane fatty acid profiles were highly similar regardless of the position of the *trans*-double bond or of the monofluoro-substituent. The exogenously supplied fatty acids generally accounted for 90 to 95 mole % of the membrane fatty acids with the products of *de novo* biosynthesis (12:0, 14:0, 16:0 and 18:0, Saito *et al.*, 1977) accounting for the remainder. The proportion of *trans*-acid to monofluoro-acid provided in the supplement was retained in the *A. laidlawii* B membranes.

Figure 19 illustrates the gel to liquid-crystalline phase transition endotherms obtained by DSC for the membrane lipids of *A. laidlawii* B supplemented with 85% 18:1 Δ 9 plus 15% monofluoro-16:0 for each particular monofluoropalmitic acid employed. These phase transition endotherms are quite distinct from those reported previously from this laboratory for *A. laidlawii* B membrane lipids highly enriched with particular fatty acids (e.g., McDonough *et al.*, 1983), in that the presence of two separate thermotropic events could be discerned. The lower temperature transition (T_1) accounted for 60 to 65% while the higher temperature transition (T_2) accounted for 35 to 40% of the total enthalpy. The separation of the two transitions ($\Delta T_1 - T_2$) was

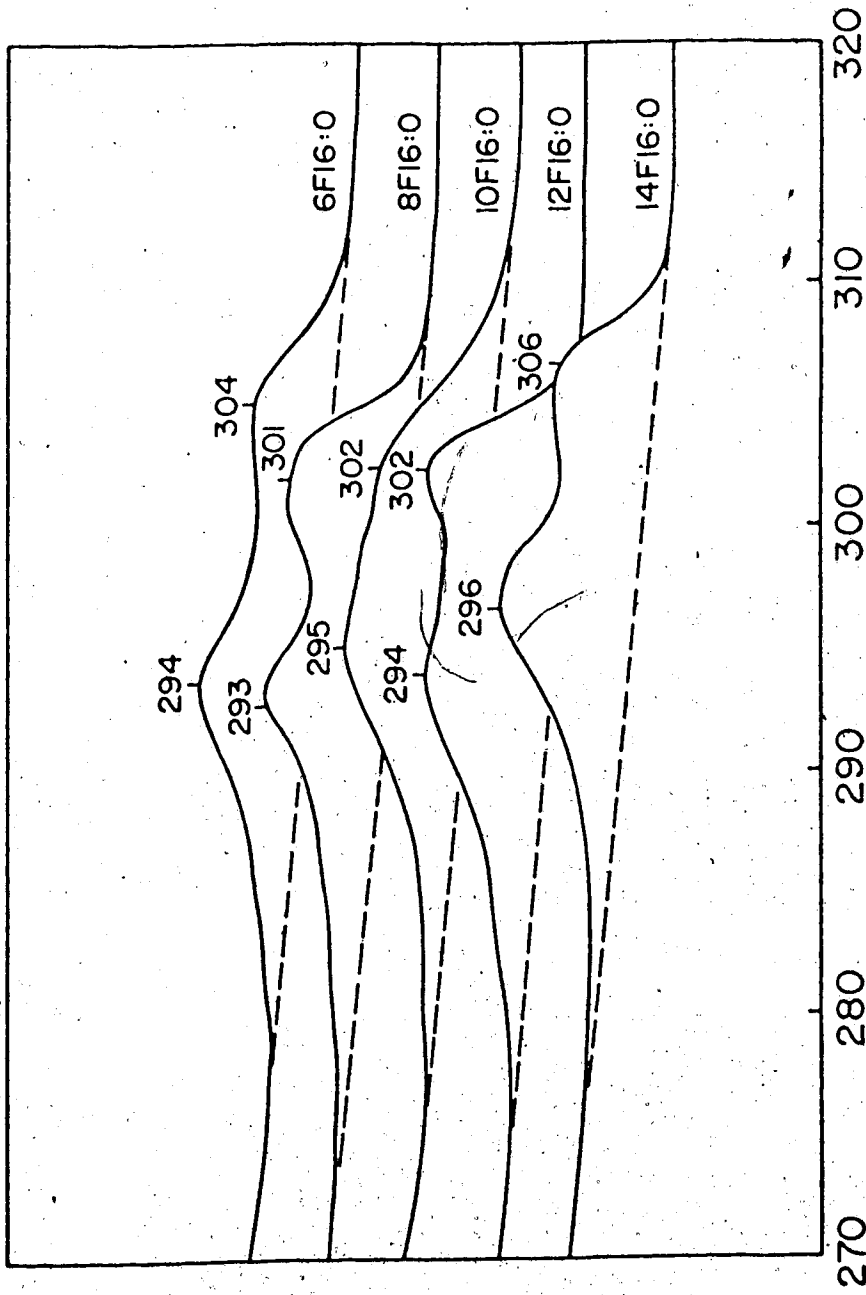
Table 5A: Composition of A. laidlawii Membranes Enriched with trans-Octadecenoic Acids Plus Monofluoropalmitic Acids.

Supplement ^a	Mole % Fatty Acid						
	12:0	14:0	16:0	18:1	xFl6:0	Other	
15% 6Fl6:0 + 85% 18:1tΔ6	0.79	1.56	2.03	80.01	11.06	4.54	
8Fl6:0	0.53	0.25	0.37	83.05	12.42	3.37	
10Fl6:0	0.27	0.19	0.41	80.36	9.31	9.46	
12Fl6:0	0.33	0.37	1.50	79.90	9.61	8.29	
14Fl6:0	0.94	0.48	1.76	80.11	10.18	6.52	
15% 6Fl6:0 + 85% 18:1tΔ9	0.57	0.18	5.27	82.50	9.50	-	
8Fl6:0	0.49	0.16	5.12	83.23	11.00	-	
10Fl6:0	0.42	0.13	5.13	82.16	12.16	-	
12Fl6:0	0.54	0.30	6.21	78.32	7.33	5.33	
14Fl6:0	1.27	0.54	5.73	78.99	7.22	5.23	
15% 6Fl6:0 + 85% 18:1tΔ11	0.32	0.43	2.23	82.89	14.13	-	
8Fl6:0	0.37	0.19	0.42	83.31	14.31	1.37	
10Fl6:0	0.25	0.22	0.22	84.23	14.67	5.42	
12Fl6:0	0.34	0.27	0.47	83.95	14.20	5.77	
14Fl6:0	0.52	0.37	0.41	84.40	14.01	6.24	

Table 5B: Composition of *A. laidlawii* Membranes Enriched with
Various Proportions of Elaidic Acid Plus Palmitic Acid

Supplement ^a	Mole % Fatty Acid			
	12:0	14:0	16:0	18:1
100% 18:1tΔ9	0.4	0.5	0.9	98.2
90% 18:1tΔ9 + 10% 16:0	0.3	0.2	11.3	88.2
80% 18:1tΔ9 + 20% 16:0	0.3	0.1	20.0	79.6
70% 18:1tΔ9 + 30% 16:0	0.3	0.1	30.7	68.9
60% 18:1tΔ9 + 40% 16:0	0.4	0.1	39.6	59.9
50% 18:1tΔ9 + 50% 16:0	0.3	0.1	49.8	49.8

^aFinal fatty acid concentration was 0.12 mM.



TEMPERATURE (°K)

Figure 19: DSC endotherms of membrane lipids of A. laidlawii B supplemented with 85 mole % 18:1 Δ 9 plus 15 mole % of various mono-fluoropalmitic acids. The scan rate was 5°K/min. (---) interpolated baseline.

largest when the monofluoro substituent was near the carbonyl headgroup or the methyl terminus and smallest when the monofluoro substituent was located near the center of the acyl chain. These characteristics, the presence of two separate thermotropic events, their relative enthalpies, and the dependence of their separation on the position of the monofluoro substituent, were observed for each of the three isomeric *trans*-octadecenoic acids employed.

Table 6 summarizes the calorimetric data for these membranes and includes, for comparison, data previously reported by Silvius *et al.* (1980) for fatty acid-homogeneous *A. laidlawii* B membranes and PC's (Silvius and McElhanev, 1979) containing various *trans*-octadecenoic acids. The P2/P1 ratio for 18:1Δ9 reported by Saito *et al.* (1977) suggests that *trans*-octadecenoic acids are six times as likely as palmitic acid to be esterified at the *sn*-2 position of the glycerol backbone of *A. laidlawii* B membrane lipids. This preference is sufficient to suggest that when the supplement is 85% 18:1tΔx plus 15% yF16:0, the membrane lipids contain approximately 30% of a mixed acid species (yF16:0/18:1tΔx) and 70% of a *di*-acid species (18:1tΔx/18:1tΔx). Since the lower temperature transition (T_c) observed here most closely corresponds to previously reported values of the phase transition temperatures of *A. laidlawii* B membranes or PC's containing *trans*-octadecenoic acids, it would seem reasonable to suggest that this transition could be attributed to a *di*-acid species, while the higher

Table 6A: Summary of Calorimetric Data on Membrane Lipids From A. laidlawii Grown in the Presence of trans-Octadecenoic Acids Plus Monofluoropalmitic Acids.

Supplement	°K							Total
	T ₁	T ₂	T _m	T ₁ -T ₂	T ₁₀₋₉₀	H ₁	H ₂	
15% 6F16:0 + 85% 18:1 Δ 6	302	313	308	11	16	62	35	
8F16:0	303	309	306	6	11	59	41	
10F16:0	303	310	305	7	11	67	33	
12F16:0	301	313	305	12	15	65	35	
14F16:0	300	311	303	11	14	69	31	
MEAN	302	311	305	9	13	64	36	
15% 6F16:0 + 85% 18:1 Δ 9	294	304	296	10	19	67	33	
8F16:0	293	301	295	8	18	59	41	
10F16:0	295	302	297	7	17	66	34	
12F16:0	294	302	296	8	16	67	33	
14F16:0	296	306	298	10	17	66	34	
MEAN	294	303	296	9	17	65	35	
15% 6F16:0 + 85% 18:1 Δ 9	297	306	302	9	15	50	36	
8F16:0	299	308	302	9	22	55	31	
10F16:0	301	303	302	2	15	-	-	
12F16:0	301	307	303	6	12	59	41	
14F16:0	302	308	303	6	6	60	40	
MEAN	300	307	302	7	13	58	42	

- a T_1 and T_2 correspond to the temperatures of the lower and higher temperature transitions respectively, T_m equals the temperature at which the transition was 50% complete, $T_1 - T_2$ equals the separation of T_1 and T_2 , ΔT_{10-90} equals the temperature range over which the transition passes from 10% to 90% of completion.
- b The total area under the transition endotherm was used as 100%. ΔH_1 or ΔH_2 equalled the area below or above the temperature minimum between T_1 and T_2 .

Table 6B: Comparison of Mean Transition Temperatures (°K) With Previously Reported Calorimetric Data.

Fatty Acid	Present Report		A. Laidlaw ^a	T _c	Phosphatidyl- choline ^b
	T ₁	T ₂			
18:1tΔ6	302	311	295.2	-	
18:1tΔ9	295	304	293.1	12.9	
18:1tΔ11	300	306	293.0	13.2	

^a Data from Silvious et al. (1980).

^b Data from Silvious and McElhanev (1979).

temperature transition (T_2) could result from the presence of a somewhat immiscible mixed-acid species containing a generally higher melting monofluoropalmitic acid. This suggestion is further supported by the observation that the proportion of the enthalpies of the two transitions, $\Delta H_1/\Delta H_2$, was generally 65/35, roughly corresponding to the expected proportions of the di-acid/mixed acid species under these conditions.

However reasonable these arguments might seem, the possibility that headgroup immiscibility as well as acyl chain immiscibility contributed to the observed phase separations cannot be excluded. DSC endotherms of *A. laidlawii* B lipids are generally somewhat asymmetric (McElhaney, 1982b) and the individual headgroup species in *A. laidlawii* B membranes, when isolated, exhibit significantly different and sometimes complex phase behaviour (Silvius *et al.*, 1980), suggesting that the asymmetric transitions observed with total *A. laidlawii* B lipid extracts may be composites of unresolved individual glycerolipid species transitions. It is noteworthy as well that the differential thermal analysis (DTA) endotherms for *A. laidlawii* B membrane lipids highly enriched with 18:1t Δ 9, show the presence of two distinct thermotropic events (McElhaney, 1974). Moreover, it is generally held that immiscibility originating in the fatty acyl chains can be correlated with the differences in melting temperatures of the species involved (Mabrey and Sturtevant, 1976). It does

not seem *a priori* that the melting temperatures of a di-18:1 Δ 9 species and a mixed 16:0-18:1 Δ 9 species should be sufficiently different as to lead to the extensive phase separation observed here. However, McElhaney (1982b) has pointed out that subtle structural features as well as differences in T_m affect phase state miscibility in model membranes.

In an attempt to distinguish the effects of headgroup vs acyl chain immiscibility I obtained the DSC endotherms at a number of scan rates (10°, 5°, 2° and 1°/min) of membrane lipids of *A. laidlawii* B containing various ratios of 16:0 to 18:1 Δ 9. These endotherms are illustrated in Figure 20 for scan rates of 5°C/min. At all proportions of 16:0 tested (0, 10, 20, 30, 40 and 50 mole % 16:0 in 18:1 Δ 9), two separate thermotropic transitions could be discerned. With increasing amounts of 16:0, three changes were noted. Firstly, the temperatures of both the lower temperature (T_1) and higher temperature (T_2) transition increased, by approximately 7°C and 14°C, respectively, as the concentration of the higher-melting palmitate was increased over the range 0 to 50 mole %. Secondly, the separation of the two transitions, $\Delta T_1 - T_2$, progressively increased from about 4°C at 100 mole % 18:1 Δ 9 to almost 11°C at 50 mole % 16:0. Thirdly, the proportion of the total transition enthalpy attributable to the higher-temperature transition, T_2 , decreased from approximately 50% at the lowest concentration of 16:0 to less than 30% at the highest

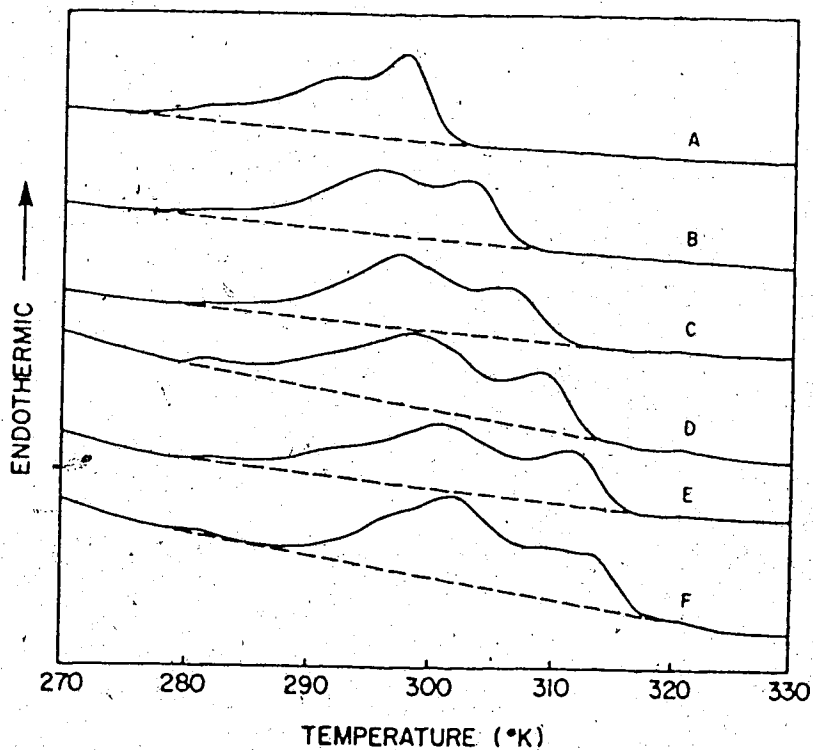


Figure 20: DSC endotherms of membrane lipids of A. laidlawii B supplemented with various proportions of 18:1tΔ9 plus 16:0 illustrated for scan rate of 5°K/min. (---), interpolated baseline. A - 100% 18:1tΔ9; B - 90% 18:1tΔ9 + 10% 16:0; C - 80% 18:1tΔ9 + 20% 16:0; D - 70% 18:1tΔ9 + 30% 16:0; E - 60% 18:1tΔ9 + 40% 16:0; F - 50% 18:1tΔ9 + 50% 16:0.

concentrations of 16:0. These observations were independent of the scan rate at which the endotherms were obtained, indicating that there were no kinetic limitations on the rates of the phase transitions which might obscure the true phase behaviour, although the temperatures of both the lower- and higher-temperature transitions appeared to increase by approximately 5°C from the lowest to the highest scan rate, due to instrumental heat-flow limitations.

Consider first the possible distribution of the two fatty acyl species, 16:0 and 18:1 Δ 9, among the major lipid classes of this organism. Saito and McElhahey (1977) observed that, although differences in the fatty acid composition of the glycolipid vs the phospholipid membrane components could be discerned when endogenous fatty acid biosynthesis was predominant, under conditions of high enrichment with exogenous fatty acids these differences disappeared. Thus, we would expect that at each ratio of 16:0/18:1 Δ 9 tested, the fatty acid composition of each of the major membrane polar lipids of *A. laidlawii* B would be approximately identical and would reflect the ratio of 16:0/18:1 Δ 9 provided exogenously. Furthermore, the P2/P1 ratio of 18:1 Δ 9 relative to 16:0 reported by Saito *et al.* (1977) was approximately 6 for each of monoglucosyl diglyceride (MGDG), diglucosyl diglyceride (DGDG) and phosphatidyl glycerol (PG) and was practically independent of the proportion of the two fatty acids compared. We would further expect, therefore, that at each ratio of

16:0/18:1tΔ9 and for each of the major polar lipid components, the palmitic acid chains will be preferentially esterified to the *sn*-1 position and that the proportion of mixed-chain (16:0, 18:1tΔ9) and *di*-acid (18:1tΔ9) species will vary directly as the ratio of 16:0/18:1tΔ9 provided exogenously, from 100% *di*-acid species at 100% 18:1tΔ9 to 100% mixed-acid species at 50/50-16:0/18:1tΔ9. These various points indicate that *a priori* we would expect little difference in the fatty acid composition or positional distribution of fatty acids within any of the major polar lipid components of *A. laidlawii* B under these experimental conditions.

Consider next the expected changes in the polar lipid composition as the ratio of 16:0/18:1tΔ9 is varied. The ratio (MGDG + DGDG)/PG has been reported to be relatively invariant with altered fatty acid composition generally averaging about a value of 3 (Silvius *et al.*, 1980). In contrast, the ratio MGDG/DGDG was found to be highly variable, equalling 0.86 at high levels of 18:1tΔ9 and increasing markedly as the degree of saturation was increased (Silvius *et al.*, 1980). Therefore one expects the neutral lipid composition to change from predominantly DGDG at 100% 18:1tΔ9 to predominantly MGDG at 50% 16:0/50% 18:1tΔ9. It can further be noted that the neutral lipids in general and DGDG in particular display complex phase behaviour in DTA studies of isolated lipids and melt at temperatures up to 10°C higher than the overall membrane

lipid phase transition (Silvius *et al.*, 1980).

Turning then to the DSC endotherms we obtained with different ratios of 16:0/18:1t Δ 9, we observe effective phase separation at all proportions. Assuming 100% *di*-acid species at 100% 18:1t Δ 9 and 100% mixed-acid species at 50% 16:0/50% 18:1t Δ 9, it must be concluded that headgroup immiscibility is responsible for the observed phase separations under these conditions, since little fatty acid compositional heterogeneity should be expected. Since the temperature of both the lower-temperature and higher-temperature transitions increased as the proportion of 16:0 was increased, it is apparent that this fatty acid was readily incorporated into the polar lipid species responsible for both transitions. Since the enthalpy of the higher-temperature transition decreased relative to the lower-temperature transition as the proportion of 16:0 increased, while it is expected that the ratio of MGDG/DGDG should increase greatly under the same conditions, it is not unreasonable to conclude that the higher temperature transition is attributable to the immiscibility of the generally higher-melting, DGDG species. This conclusion further suggests that headgroup immiscibility is responsible for the thermotropic behaviour of membrane lipids containing 85% 18:1t Δ x plus 15% yF-16:0. Thus the monofluoropalmitic acids are probably not particularly enriched in the polar lipid species responsible for either the lower- or higher-temperature transition but are most likely evenly

distributed among all species, as concluded previously from direct experimentation (McDonough *et al.*, 1983). As a basis for comparison among the various isomeric *trans*-octadecenoic studied here, I have therefore used the centre of the entire phase transition rather than one or the other of the two distinct thermotropic events.

¹⁹F-NMR order profiles are illustrated in Figure 21 for each of the three isomeric *trans*-octadecenoic acids studied at temperatures above (310°K), just below (289°K) and well below (279°K) the gel to liquid-crystalline phase transition. In the liquid-crystalline state at 310°K, the order profiles were qualitatively similar for each isomeric *trans*-octadecenoic acid, with a region of relatively constant order preceding a region of progressively declining order towards the methyl terminus of the acyl chain. Nevertheless, it was possible to observe a local disordering effect of the *trans*-double bond in the ¹⁹F-NMR order profile as reported previously (Macdonald *et al.*, 1983). The order profile of 18:1tΔ6 most closely resembled that of a straight-chain saturated fatty acid such as 15:0 or 16:0. Given the inequality of the penetration into the bilayer of the *sn*-1 and *sn*-2 esterified acyl chains and the fact that the methylene segment nearest the carbonyl headgroup were not monitored, this result is not surprising. Furthermore, it is expected that chain-packing densities should increase substantially nearer the lipid polar headgroup so that local ordering or disordering effects of particular substituents

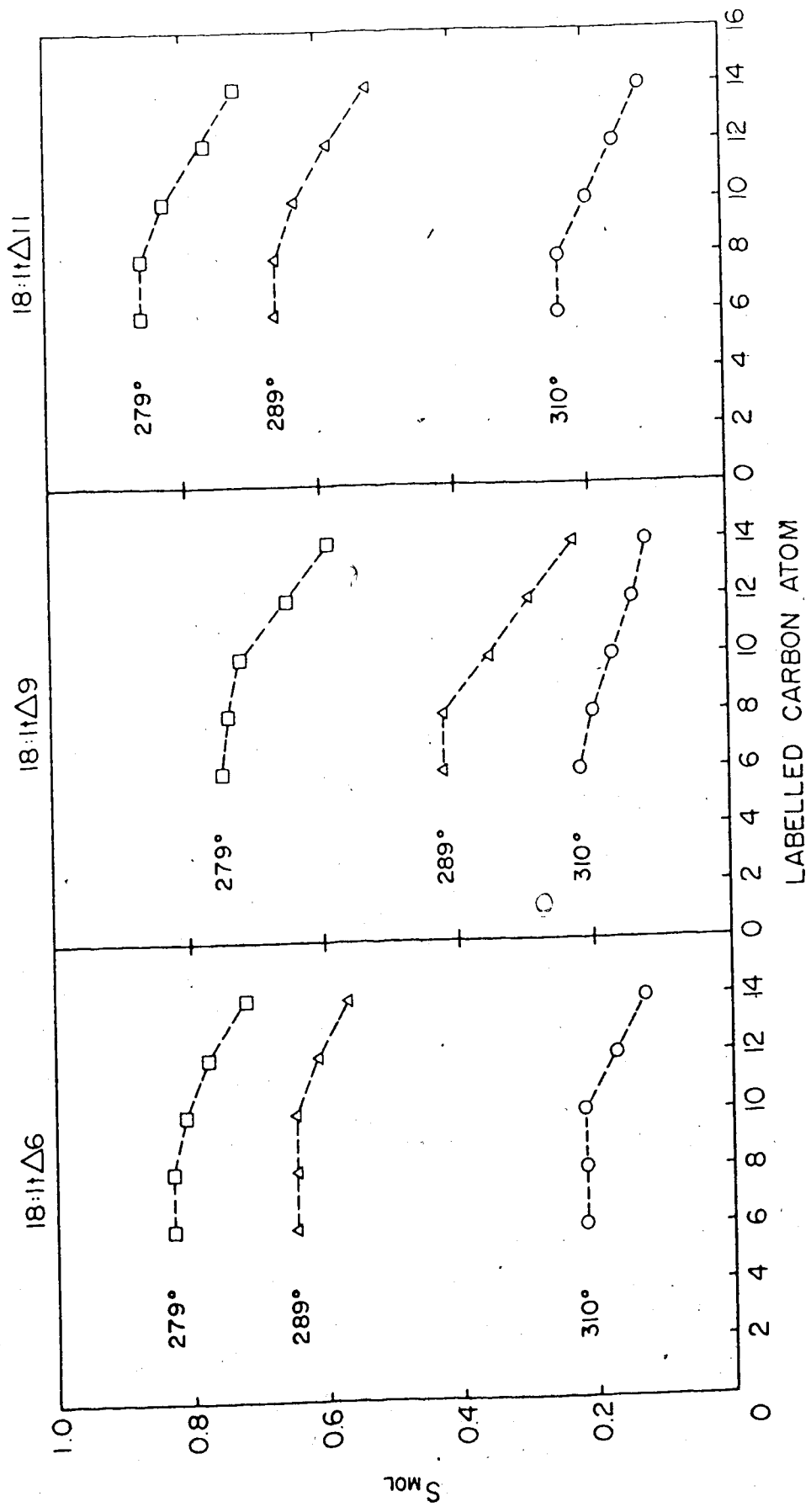


Figure 21: ^{19}F -NMR order profiles of *A. laidlawii* B membranes enriched with various isomers of trans-octadecenoic acid plus monofluorinated palmitic acids acquired at temperatures above (310°K), just below (289°K) and far below (279°K) the lipid phase transition.

should be less readily manifest. The local disordering effect of the *trans*-double bond was most apparent in the case of 18:1tΔ9 but was also apparent in the order profile obtained in the presence of 18:1tΔ11. Seelig and Waespe-Sarčević (1978) described the ²H-NMR order profile of 1-16:0, 2-18:1tΔ9 PC and found no differences between the order profile of the elaidoyl chain and that of a palmitoyl chain in DPPC. However, the same may be said of the *cis*-double bond after correction for geometric considerations, despite its local ordering effect on a neighbouring chain (Seelig and Seelig, 1977).

At a temperature just below the phase transition (289°K), orientational order increased substantially at all positions for all isomeric *trans*-octadecenoic acids. Despite the predominance of gel-state lipid at this temperature the values of the order parameters had not yet approached the theoretical maximum of 1.0. In addition, a head-to-tail gradient in orientational order was still apparent at 289°K. The much greater disorder of the 18:1tΔ9 isomer at 289°K may be simply due to the fact that, at this temperature, membranes enriched with this isomer were closer to their phase transition than either of the other isomers (see Table 6).

At 279°K each of these membrane preparations were well below their respective gel to liquid-crystalline phase transitions. The order profiles at 279°K were characteristic of a highly ordered lipid state with individual values of

S_{m0} , approaching but not achieving the theoretical maximum. Despite this uniformly high ordering, the order profiles indicated that, even far below the phase transition, in the presence of a *trans*-double bond a head-to-tail gradient of orientational order still existed. In keeping with the previously reported conclusion that certain structural substituents more readily manifest a disordering effect in the gel-state when they are located further from the carbonyl headgroup of the fatty acid, it could be discerned that the head-to-tail gradient of order was more pronounced in the presence of 18:1 Δ 9 or Δ 11 than in the presence of 18:1 Δ 6. In fact, the gradient of order in the presence of 18:1 Δ 11 was greater than that of 18:1 Δ 6 even at 289°K. Thus the *trans*-double bond was apparently able to somewhat disrupt or prevent the assumption of a uniformly highly ordered state. This effect was therefore manifest as a gradient of disorder that remained in the gel-state. It is pertinent to note that the gel-state order profiles of straight-chain saturated fatty acids obtained by ¹H-NMR (Allegrini *et al.*, 1983) or ¹⁹F-NMR are relatively flat, with very little head-to-tail decrease in orientational order. This disruption due to a *trans*-double bond is, however, much less pronounced than that observed in the gel-state in the presence of a *cis*-double bond. The implication is then that the decrease in the temperature of the phase transition in the presence of a *cis*- or *trans*-double bond may be qualitatively correlated with the

extent of disordering in the gel state attributable to a particular substituent.

Figure 22 compares the chain-average order parameters for each of the three isomers of *trans*-octadecenoic acid at the reduced temperature T_r . The value of T_r was calculated using the temperatures corresponding to the middle of the entire phase transition listed in Table 6 as T_m . When this comparison was made, it became apparent that the *trans*-octadecenoic acids behaved very similarly as a class with respect to the phase transition. In the liquid-crystalline state overall order increased in an approximately linear fashion with increasing proximity to the phase transition. Once the center of the phase transition was traversed, overall order increased rapidly but only approached maximal values when the phase transition was greater than 90% complete. When an analogous comparison was made of the overall order of an isomeric series of *cis*-octadecenoic acids at a reduced temperature, the dependence of order on the phase transition was nearly identical to that observed with isomeric *trans*-octadecenoic acids in Figure 22. Thus, the lipid phase transition appears to be the preeminent effector of overall order independent of the configuration of the double bond (*cis* or *trans*) or its location along the acyl chain.

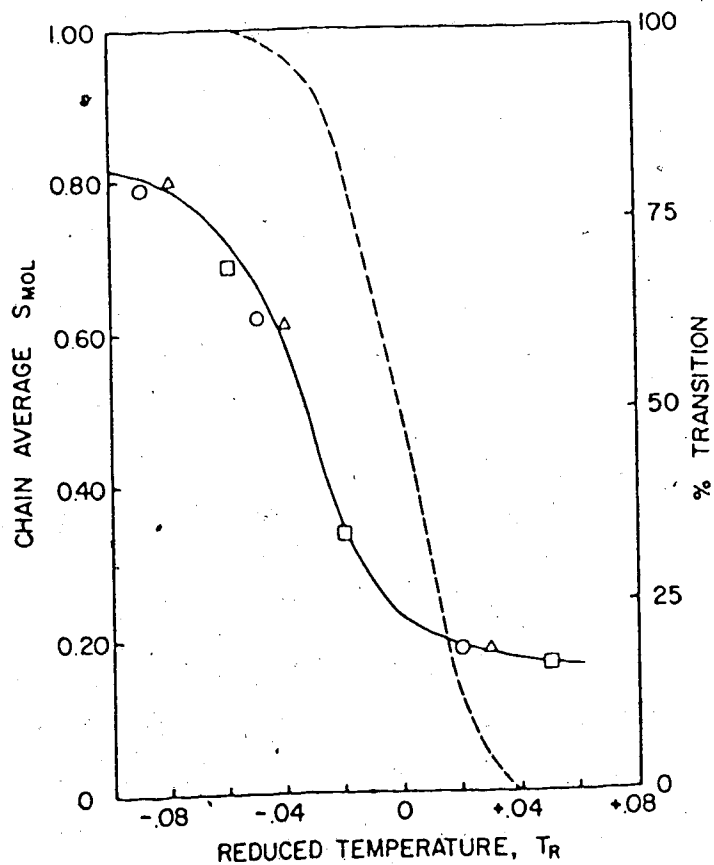


Figure 22: Normalization of the chain average order parameter with respect to the particular phase transition temperature for each case of enrichment with a particular isomer of trans-octadecenoic acid. Chain average order was calculated as described in the text. The mean value of T_m from Table 6 was used to calculate T_R . Dashed line shows the % transition completed as a function of the reduced temperature. Circles, 18:1tΔ6; Squares, 18:1tΔ9; triangles, 18:1tΔ11.

Methyl *ISO*- and *Anteiso*-Branched Fatty Acids

The methyl *iso*- and *anteiso*-branched saturated fatty acids are found as components of the membrane lipids of many prokaryotic microorganisms and in some higher organisms, and are the predominant fatty acyl species in a variety of bacteria (Polgar, 1971; Kaneda, 1977). Branched-chain fatty acids are able to support the growth of several unsaturated fatty acid auxotrophic bacteria apparently by mimicking the properties of unsaturated fatty acids (Rodwell and Petersen, 1971; Silbert *et al.*, 1973; Silvius and McElhaney, 1978a). Both model membrane (Silvius and McElhaney, 1979, 1980a) and natural membrane (McElhaney, 1974; Blume *et al.*, 1978; Silvius *et al.*, 1980) systems exhibit decreased gel to liquid-crystalline phase transition temperatures, relative to membranes containing straight-chain saturated fatty acids, when they are enriched in branched-chain fatty acyl species. Despite their widespread occurrence and their probable role as membrane "fluidizing" agents analogous to the *cis*-unsaturated fatty acids, it is only recently that the physical properties of methyl-branched fatty acids have begun to receive attention. Monolayer studies of PC's containing methyl branched-chain fatty acids indicate that *iso*-branching and particularly *anteiso*-branching reduce the temperature of the liquid-expanded to liquid-condensed transition and also increase the area occupied per molecule in the liquid-condensed state (Kannenburg *et al.*, 1983). Differential scanning calorimetry (DSC) studies have

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demonstrated that PC's containing methyl-branched fatty acids can exhibit complex phase behaviour (Lewis *et al.*, 1984). The multiple endothermic events observed calorimetrically correspond to solid-solid as well as solid-liquid thermotropic transitions of the methyl branched chain-containing PC's. Fourier transform infrared (FTIR) spectroscopic studies indicate that these solid-solid thermotropic transitions may have properties in common with both the subtransition and the pretransition exhibited by linear saturated PC's (Madec *et al.*, 1984). Fluorine-19 nuclear magnetic resonance (^{19}F -NMR) spectroscopic studies of membranes of *A. laidlawii* B enriched with methyl branched-chain fatty acids have shown that, in the liquid-crystalline state, these fatty acyl species have an apparent overall ordering effect on a monofluoropalmitoyl nuclear spin probe (Macdonald *et al.*, 1983). To date, the effects of methyl branched-acyl chains on conformation in the hydrocarbon interior of the lipid bilayer in both the gel-state and the liquid-crystalline state have not been investigated. Towards this end I initiated ^{19}F -NMR studies of *A. laidlawii* B membranes highly enriched in either a straight-chain saturated, a methyl *iso*-branched or a methyl *anteiso*-branched fatty acid plus small quantities of various isomeric monofluoropalmitic acids as nuclear spin probes.

Table 7 lists the fatty acid composition for each case of enrichment with either 15:0, 16:0*i* or 16:0*ai* plus a particular isomeric MFPA. In all cases the fatty acids

Table 7: Fatty Acid Composition of A. laidlawii B Membrane Lipids Enriched With 15:0, 16:0i or 16:0ai plus Various Monofluoropalmitic Acids

Supplement	Mole % Fatty Acid						
	15:0	16:0i	16:0ai	xFl6:0	Other		
85% 15:0 + 15%	6Fl6:0	93.3	--	--	6.3	0.4	
	8Fl6:0	92.3	--	--	7.2	0.5	
	10Fl6:0	95.9	--	--	4.0	0.1	
	12Fl6:0	97.0	--	--	2.7	0.3	
	14Fl6:0	94.4	--	--	5.2	0.4	
85% 16:0i + 15%	6Fl6:0	--	87.7	--	12.0	0.3	
	8Fl6:0	--	92.2	--	7.5	0.3	
	10Fl6:0	--	90.8	--	8.8	0.4	
	12Fl6:0	--	92.8	--	6.7	0.5	
	14Fl6:0	--	87.7	--	11.9	0.4	
85% 16:0ai + 15%	6Fl6:0	--	--	89.2	10.5	0.3	
	8Fl6:0	--	--	87.0	12.8	0.2	
	10Fl6:0	--	--	89.4	10.1	0.5	
	12Fl6:0	--	--	89.0	10.7	0.3	
	14Fl6:0	--	--	88.4	11.1	0.5	

provided exogenously accounted for greater than 99% of the membrane lipid fatty acids. The products of *de novo* fatty acid biosynthesis in *A. laidlawii* B (12:0, 14:0, 16:0 and 18:0, Saito *et al.*, 1977) were conspicuously absent as expected for cells grown in the presence of avidin, each individually accounting for no greater than 0.1 mole % of the total membrane fatty acids. With the exception of cells enriched with 15:0, the ratio of the two fatty acids provided in the supplement was carried over reasonably well into the membrane lipids, indicating little if any selectivity towards either of the two fatty acyl species. However, in the case of enrichment with 15:0, the mole % of monofluoropalmitic acid found in the membrane lipids was significantly less than that provided exogenously. Since 15:0 represents the upper chain-length limit of the linear, saturated fatty acyl family which will support the growth of *A. laidlawii* B in the presence of avidin (Silvius and McElhanev, 1978a), extensive incorporation of longer linear saturated fatty acids such as monofluoropalmitic acids would be detrimental to the maintenance of a properly fluid membrane and hence to cell growth and viability. Therefore, the organism may selectively incorporate proportionately fewer monofluoropalmitic acids. Regardless, the levels of monofluoropalmitic acid incorporation were adequate in all cases for the acquisition and analysis of ^{19}F -NMR spectra.

DSC endotherms of the *A. laidlawii* B membrane polar lipid fraction were obtained for each case of enrichment

with a particular isomeric MFPA plus either of 15:0, 16:0i or 16:0ai. Figure 23 illustrates the DSC endotherms obtained in the case of enrichment with 16:0i for each of the isomeric MFPA's employed. The endotherms were typically broad, somewhat asymmetric and generally similar to those reported previously by this laboratory for *A. laidlawii* B membrane lipids (McDonough *et al.*, 1983). The variation in T_m from isomer to isomer of MFPA was minimal, generally being less than $\pm 1^\circ\text{C}$ from the average. Table 8 summarizes the calorimetric data for all cases of enrichment studied here and provides, for comparative purposes, previously reported thermotropic data for PC's (Silvius and McElhaney, 1979, 1980) and *A. laidlawii* B membrane lipids (Silvius *et al.*, 1980) containing essentially 100 mole % 15:0, 16:0i and 16:0ai. It is evident from this table that the gel to liquid-crystalline phase transition temperature was somewhat higher for the *A. laidlawii* B membrane lipids than the corresponding phase transition temperature in the PC. The presence of approximately 10 mole % MFPA in the *A. laidlawii* B membrane lipids further increased the phase transition temperature of the *A. laidlawii* B membrane lipids, as expected for these higher-melting fatty acids. Nevertheless, the same trend of progressively decreasing phase transition temperatures from 15:0 to 16:0i to 16:0ai was evident in the PC's, *A. laidlawii* B fatty acid-homogeneous membrane lipids and *A. laidlawii* B membrane lipids containing small amounts of MFPA's as well.

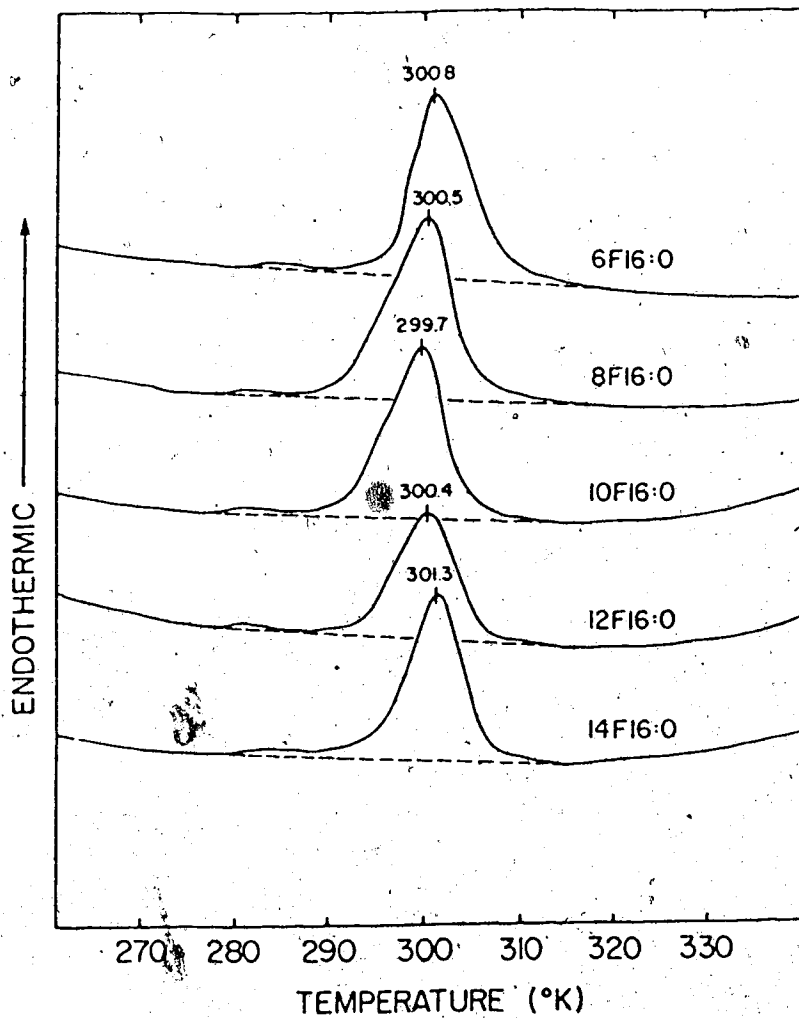


Figure 23: Lipid phase transition endotherms obtained by differential scanning calorimetry of the membrane polar lipid fraction from A. laidlawii B grown in the presence of 5 mole % 16:0i plus 15 mole % various isomeric monofluoropalmitic acids. The scan rate was 5°C/min. Dashed line corresponds to the interpolated baseline.

Table 8: Thermotropic Properties of A. laudlawii B Membrane Polar Lipids Enriched With 15:0, 16:0i or 16:0ai Plus Various Monofluoropalmitic Acids

Supplement ^c	T _m (°C)	T ₁₀₋₉₀ (°C) ^a	T _m (A. laudlawii homogeneous) (°C) ^b	T _F PC (°C) ^c
85% 15:0 + 15%	6F16:0	38.11	8.75	
	8F16:0	41.34	12.20	
	10F16:0	39.99	8.51	
	12F16:0	40.27	9.07	
	14F16:0	40.91	10.73	
<u>Average</u>	40.12	9.85	36.7	34.2
85% 16:0i + 15%	6F16:0	27.84	9.43	
	8F16:0	27.48	11.07	
	10F16:0	26.68	9.98	
	12F16:0	27.35	10.32	
	14F16:0	28.30	10.01	
<u>Average</u>	27.53	10.18	21.8	22.5
85% 16:0ai + 15%	6F16:0	11.07	13.51	
	8F16:0	11.40	11.38	
	10F16:0	12.40	12.81	
	12F16:0	11.84	10.94	
	14F16:0	13.18	11.60	
<u>Average</u>	11.98	12.05	4.1	-3.6

^a ΔT₁₀₋₉₀ corresponds to the temperature range over which the transition passes from 10% to 90% of completion.

^b Data from Silvius et al. (1980).

^c Data from Silvius and McElhanev (1979, 1980a).

Typical experimental ^{19}F -NMR spectra of a MFPA incorporated into the membrane lipids of *A. laidlawii* B and the corresponding simulated spectra are shown in Figure 24 for *A. laidlawii* B membranes containing 6F16:0, plus one of either 15:0, 16:0i or 16:0ai, at temperatures both above and below the particular lipid phase transition. The values of the input parameters required to simulate the spectra are shown as well. The extensive broadening of the ^{19}F -NMR spectrum which occurred upon decreasing the temperature through the gel to liquid-crystalline phase transition is readily apparent. It is clear as well that the ^{19}F -NMR spectra were adequately simulated at all temperatures and in both the gel and liquid-crystalline phases. An examination of the ratio Δ_0/S_{m0} in the gel-state ^{19}F -NMR spectra indicates that at approximately equal temperatures below their respective lipid phase transitions, these different acyl chain structures have assumed gel-states which exhibit markedly different physical behaviour. The methyl branched chain-containing membranes failed to achieve the highly orientationally ordered gel-state exhibited by linear saturated fatty acyl chains, despite a comparable or greater increase in the value of Δ_0 , indicative of the decreased rate of lipid lateral diffusion in the gel-state.

Figure 25 depicts the ^{19}F -NMR order profiles of *A. laidlawii* B membranes enriched with either 15:0, 16:0i or 16:0ai obtained over a range of temperatures spanning the gel to liquid-crystalline phase transitions. These

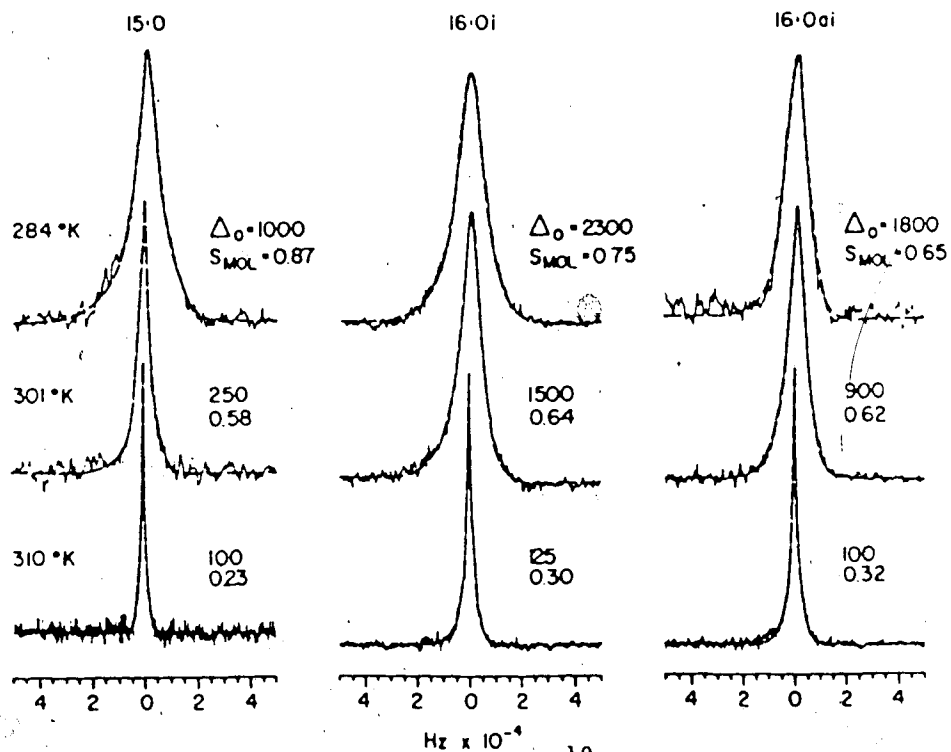


Figure 24: Experimental and simulated ^{19}F -NMR spectra of membranes of *A. laidlawii* B grown in the presence of 15 mole % 6F16:0 plus 85 mole % 15:0 (284, 301 and 310°K), 16:0i (269, 284 and 301°K), or 16:0ai (254, 270 and 186°K). The numbers in brackets represent the temperatures, from top to bottom, at which the experimental spectra were acquired.

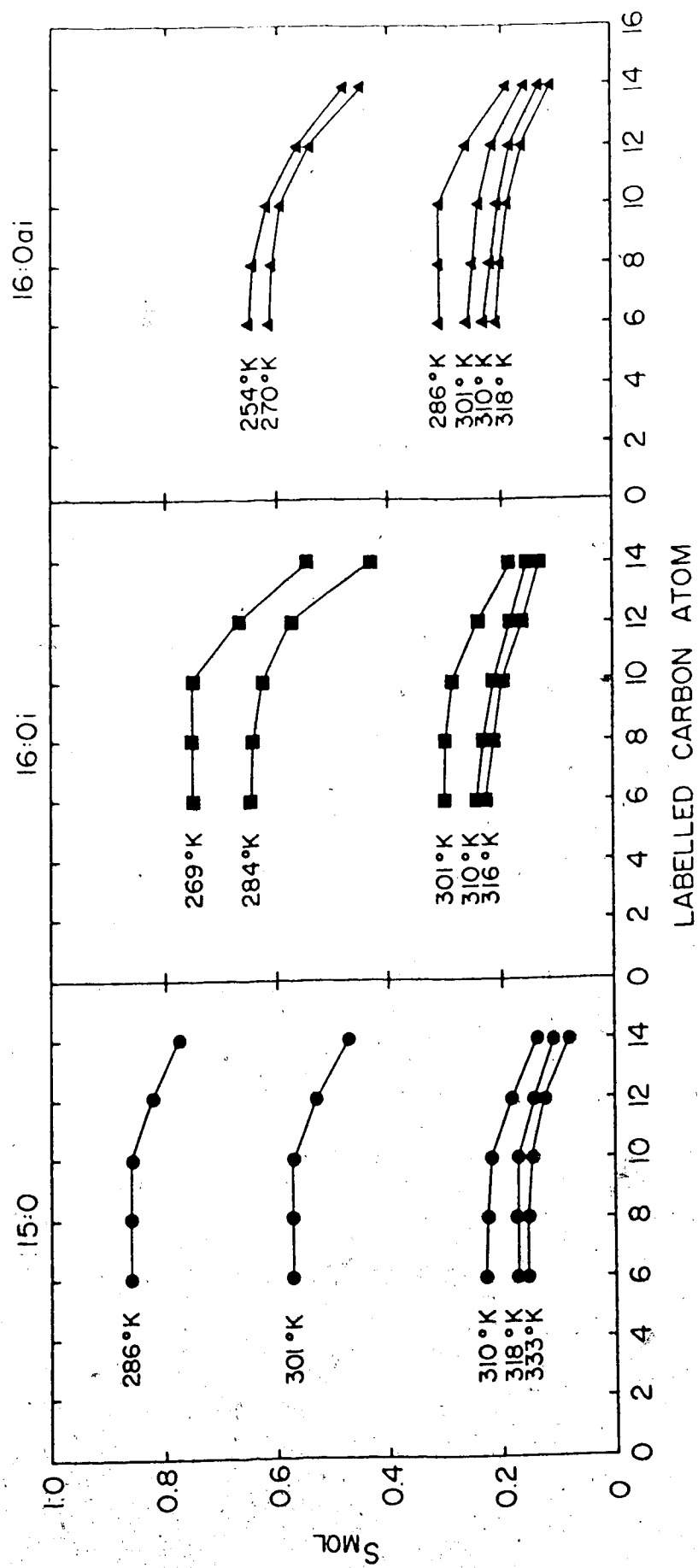


Figure 25: ^{19}F -NMR orientational order profiles of membranes of
 A. Laidlawii B enriched with 85 mole % 15:0, 16:0i or 16:0ai plus
 15 mole % various MFPA.

temperatures were chosen to permit comparisons at certain values of absolute temperature as well as at certain values of the reduced temperature (see below) above or below the particular phase transition temperature. In the liquid-crystalline state, the ^{19}F -NMR order profiles exhibit a region of relatively constant order followed by a decline in orientational order towards the methyl end of the fatty acyl chain. The effects of methyl *iso*- or *anteiso*-branching on the ^{19}F -NMR order profile were marginal in the liquid-crystalline state, although a local ordering effect of methyl-branching could be discerned as a decrease in the steepness of the terminal order gradient by comparison with the linear saturated situation, as reported previously (Macdonald *et al.*, 1983). The elasticity of the fatty acyl chains in the liquid-crystalline state should permit them to accommodate the presence of structural substituents such as a methyl *iso*- or *anteiso*-branch without drastically altering the character of the orientational order profile. It has become increasingly apparent that the order profile is relatively refractory to change in the liquid-crystalline state despite alterations to fatty acyl chain chemistry. Previously, ^{19}F -NMR results have demonstrated that the hydrocarbon chain orientational order profiles in membranes of *A. laidlawii* B are highly similar in the liquid-crystalline state whether those membranes were enriched with straight-chain saturated fatty acids such as 15:0 or 16:0 or any of an isomeric series of *cis*-octadecenoic acids or

trans-octadecenoic acids or with methyl-branched fatty acids such as 16:0i or 16:0ai. Results obtained with specifically deuterated fatty acids via $^2\text{H-NMR}$ also indicate that the liquid-crystalline order profile is relatively invariant despite changes in fatty acyl structure. The orientational order profiles of PC's containing *cis*-unsaturated fatty acids were very similar to those obtained with *trans*-unsaturated fatty acids after correction for geometric considerations and these were in turn very similar to the order profiles of straight-chain saturated fatty acids (Seelig and Waespe-Sarčević, 1978). These results were confirmed by $^2\text{H-NMR}$ studies in *E. coli* (Gally *et al.*, 1979) and *A. laidlawii* B (Stockton *et al.*, 1977; Rance *et al.*, 1980). The one exception to this generalization appears to be the cyclopropyl ring-substituted fatty acids where, even after correction for geometric considerations, the $^2\text{H-NMR}$ order profiles in both model (DuFourc *et al.*, 1983) and biological membranes (Jarrell *et al.*, 1983) indicate that the cyclopropyl substituent experiences a far greater degree of orientational ordering than any other portion of the fatty acyl chain.

With decreasing temperature, orientational order increased slowly and the character of the order profiles remained relatively constant until the lipid phase transition was traversed. As the proportion of gel state lipid increased, overall order increased profoundly and the order profiles in the presence of different fatty acyl chain

structures began to acquire distinct dissimilarities. With the straight-chain acid, 15:0, a high degree of ordering was achieved with individual values of S_{m01} , approaching the theoretical maximum. In addition, it can be discerned that the gradient of order so prominent in the liquid-crystalline state (i.e., at 333°K, the value of S_{m01} at the 14-position was approximately 50% of the value at the 6 position) was nearly absent in the gel-state (i.e., at 280°K, the value of S_{m01} at the 14-position was over 90% of the value at the 6-position). Consequently the configuration of the MFPA chain in the presence of 15:0 in the gel-state may be described as highly ordered, approaching but not as yet achieving an all-*trans* conformation, with any residual *gauche* rotational isomers distributed with approximately equal probability along most of the length of the fatty acyl chain. ³¹P-NMR gel-state order profiles of *A. laidlawii* B membranes highly enriched with 16:0 were also characteristically flat with individual values of the order parameter approaching the theoretical maximum. Allegrini *et al.* (1983) studied the gel-state ¹H-NMR spectra of specifically deuterated palmitic acids mixed equimolar with 1-palmitoyllyso PC. These compounds together assume a bilayer structure, most probably via the formation of a functional dimer of lysophospholipid and free fatty acid. The free deuterated palmitic acid experiences a higher degree of rotational freedom in the gel-state than would DPPC, which permits the characterization of its

configuration in the gel-state in terms of orientational order parameters. The values of S_{m0} , obtained in the gel-state nearly approached unity for positions C-2 to C-13 and thereafter somewhat decreased towards the acyl chain methyl terminus. Fluorine and deuterium techniques apparently agree regarding the conformation assumed by a linear saturated fatty acyl chain in the gel-state.

The inclusion of a methyl-branch substituent had two immediately apparent consequences for the gel-state order profile of MFPA. In the presence of either a methyl *iso*- or *anteiso*-branched fatty acid in the gel-state, a large head-to-tail gradient of orientational order remained, and the overall order achieved at comparable temperatures below the lipid phase transition was significantly less than was observed in the presence of the linear, saturated 15:0.

The gradient of order which remained in the gel-state in the presence of methyl branched-chain substituents indicates that these structures are capable of disrupting the gel-state acyl chain packing in their immediate vicinity. A similar gradient of order was observed via ^{19}F -NMR in the gel-state in the presence of isomers of *cis*-octadecenoic acid with the site of unsaturation near the methyl terminus of the acyl chain. Since the ^{19}F -NMR order profiles in the gel-state in the presence of isomers of *cis*-octadecenoic acid with the site of unsaturation near the carbonyl headgroup resembled those obtained in the gel-state in the presence of linear, saturated fatty acids, it was

concluded that, even in the gel-state, a head-to-tail gradient in the stringency of packing restrictions was still present. The results obtained here with methyl *iso*- and *anteiso*-branched fatty acyl species confirm that structural substituents located near the fatty acyl chain methyl terminus are capable of locally disrupting gel-state chain packing.

The second consequence of the inclusion of methyl branched structures, which is that the overall order achieved in the gel-state was far less than was achieved in the presence of 15:0, is more readily apparent when the chain average order parameters are plotted versus the acquisition temperature for the three cases of enrichment with either 15:0, 16:0i or 16:0ai, as shown in Figure 26. The chain-average order was again simply the numerical average of the five values of S_{m0} , obtained at any one temperature for any one case of enrichment. While such a data reduction will obscure differences in the characters of the order profiles, it does provide a convenient measure of overall orientational order in a given situation. Several important features are illustrated in Figure 26. At a temperature which was above the lipid phase transition of all three enriched membranes, the overall order was generally very similar regardless of the particular fatty acyl chain structure examined. For any one measuring temperature at which all fatty acyl chain structures were in the liquid-crystalline state, overall order was an

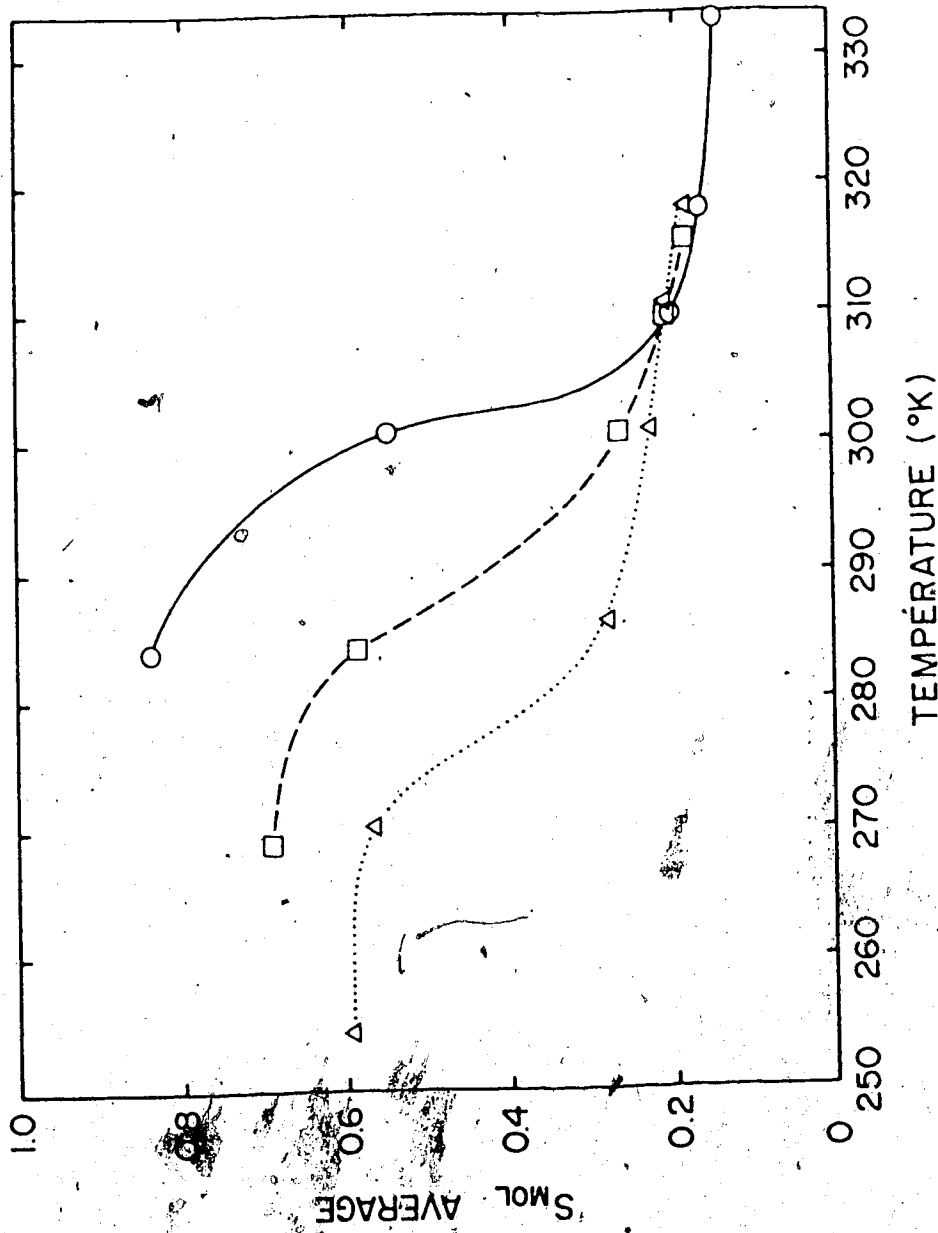


Figure 26: Chain average order versus acquisition temperature for the cases of enrichment with 15:0 (circles), 16:01 (squares) or 16:0a1 (triangles).

approximately linear function of $(T - T_m)$ and was relatively independent of the particular structure examined. For example, at 310°K , for the case of 16:0i, $S_{m0i} = 0.207$ while $T - T_m = 9^\circ\text{C}$, and for the case of 16:0ai, $S_{m0ai} = 0.193$ while $T - T_m = 23^\circ\text{C}$. Therefore, overall order generally increased with increasing proximity to the lipid phase transition. The slope of the $(T - T_m)$ dependency of the overall order increases with decreasing temperature so that at 310°K the effect is really quite minimal and, as was pointed out above, the overall order observed at 310°K was quite similar regardless of whether 15:0, 16:0i or 16:0ai was examined. At a sufficiently high temperature overall order should become independent of $(T - T_m)$ and depend only on the measuring temperature, provided one is observing exclusively the liquid-crystalline state.

As the acquisition temperature was lowered the overall orientational order increased profoundly in a given case of enrichment when, and not until, the particular lipid phase transition was encountered. Clearly the lipid phase transition was the greatest single affecter of orientational order regardless of the particular fatty acyl chain structure or the absolute temperature of the phase transition. Nevertheless, membranes containing methyl branched-chain fatty acids appeared to be incapable of assuming a gel-state as highly ordered as that observed in the presence of 15:0.

This effect is again apparent when the orientational order data are normalized with respect to the particular phase transition temperature via the introduction of a reduced temperature, T_R . Figure 27 depicts the chain-average order parameter as a function of the reduced temperature, T_R , for each case of enrichment with either 15:0, 16:0i or 16:0ai. The T_m (*A. laidlawii* B homogeneous plus MFPA) data of Table 8 were used to calculate T_R . All fatty acids showed a marked increase in orientational order as the proportion of gel-state lipid increased, indicating again that the lipid phase transition was the preeminent affecter of overall orientational order. Nevertheless, at equal values of T_R below the lipid phase transition, orientational order decreased in the progression 15:0 > 16:0i > 16:0ai. Evidently these methyl-branched substituents were capable of disrupting gel-state chain packing in more than a local sense and, in fact, prevented the assumption of the overall highly ordered, nearly all-*trans* state characteristic of straight-chain saturated fatty acids in the gel-state. It is informative to note that the temperatures of the lipid phase transitions in the presence of these fatty acids decrease in the same progression as their relative overall order in the gel-state, that is: 15:0 > 16:0i > 16:0ai. The implication is then that gel-state disordering or instability leads to a lower lipid phase transition temperature and that alterations to gel-state stability can be produced by altering fatty acyl chain structure.

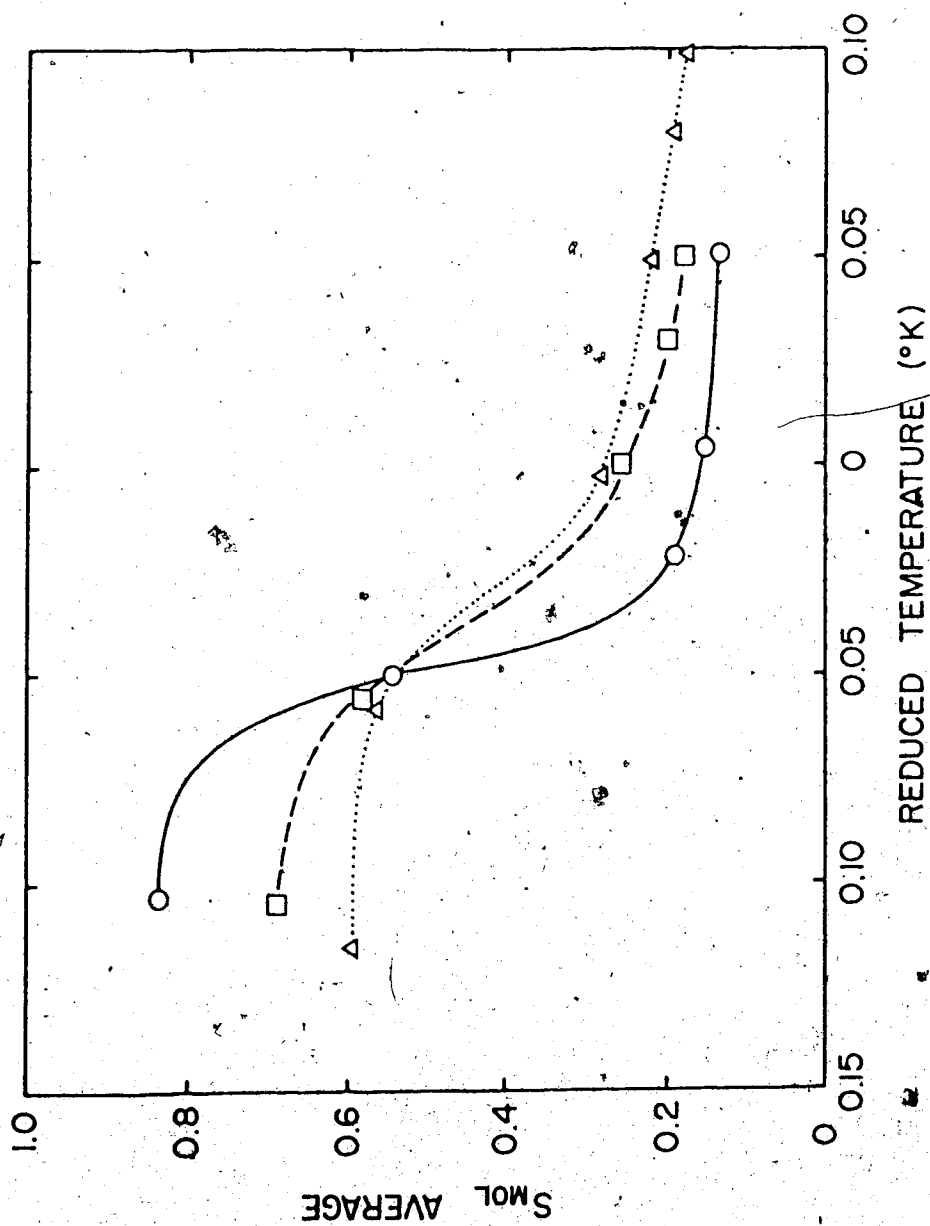


Figure 27: Chain average order versus reduced temperature for the cases of enrichment with 15:0 (circles), 16:01 (squares) or 16:0a1 (triangles).

There are a number of lines of evidence which indicate that methyl branched-chain fatty acids assume a loosely packed gel-state. Natural membranes enriched with branched-chain fatty acids exhibit unusual X-ray diffraction properties below their phase transition temperatures (Haest *et al.*, 1974; Legendre *et al.*, 1980). The sharp 4.2 Å reflection, which is associated with reflections from the closely packed hydrocarbon chains in gel-state lipid, is replaced by a broader reflection with a spacing of 4.3 - 4.4 Å in these membranes. Pig pancreatic phospholipase A₂, which cannot hydrolyze gel-state phosphatidylglycerol in *A. laidlawii* B membranes enriched with straight-chain saturated or unsaturated fatty acids, can attack this phospholipid in membranes enriched in branched-chain fatty acids at temperatures well below the lipid phase transition (Bouvier *et al.*, 1981). Moreover, the lateral segregation of integral membrane proteins into protein-rich domains, which is normally observed by freeze-fracture electron microscopy at temperatures below the lipid phase transition, does not occur in *A. laidlawii* B membranes artificially enriched in branched-chain fatty acids (Haest *et al.*, 1974; Silvius and McElhaney, 1980). Finally, monolayer studies indicate that PC's containing methyl *iso*- and *anti-iso*-branched fatty acids reduce the temperature of the liquid-expanded to liquid-condensed transition and increase the molecular area occupied per PC in the liquid-condensed state (Kannenburg *et al.*, 1983). Thus the results obtained in the present

study confirm those obtained in earlier work.

It is evident in Figure 27 that, at values of T_R above the lipid phase transition, the overall order decreases in the progression $16:0ai > 16:0i > 15:0$. This observation suggests that these methyl branched-chain fatty acids assume a more highly ordered liquid-crystalline state than does a linear saturated fatty acid. This result was previously rationalized in terms of fatty acyl chain-packing effects arising from differences in the cross-sectional area occupied by these different chain structures (Macdonald *et al.*, 1983). Recent monolayer studies by Kannenberg *et al.*, (1983) indicate, however, that methyl-branched and linear saturated fatty acids occupy approximately equal cross-sectional areas in the monolayer in the liquid-expanded state. Packing considerations seem insufficient to explain the more highly ordered liquid-crystalline state assumed by these methyl branched structures at equal values of T_R . The simplest explanation may be that at equal values of $T_R > 0$, one is actually making comparisons across a wide range of absolute temperature. From Figure 26 it can be seen that, at 310°K, 15:0, 16:0i and 16:0ai are almost equally ordered. As the temperature is decreased the orientational order of first 15:0 and then 16:0i rises dramatically as the lipid phase transition is encountered. However, over the same temperature range and in the absence of any phase change, the orientational order in the presence of 16:0ai increases progressively and significantly. This dependence of

orientational order on absolute temperature in the liquid-crystalline state offers the most probably explanation for the apparently higher ordering of methyl branched-chain fatty acids at constant values of $T_r > 0$.

A Comparison of *cis* and *trans* Monounsaturations and Cyclopropane Ring Substitution

Cyclopropane fatty acids are common membrane lipid constituents in many gram-negative and a few gram-positive bacteria (Christie, 1970; 1973). Biosynthesis of the cyclopropane ring occurs by the introduction, via S-adenosyl-methionine, of a methylene bridge across the double bond of a *cis*-unsaturated homologue. Cyclopropane fatty acids have been postulated to be more stable replacements for unsaturated fatty acids (Christie, 1970; Cronan and Vagelos, 1972). Generally, in those organisms in which they are found, the proportion of cyclopropane fatty acids increases at later stages of growth.

The overall physical properties of *cis*-cyclopropane and *cis*-unsaturated substituted lipids are rather similar. The molecular packing of lipids containing cyclopropane fatty acids and those containing monounsaturated fatty acids are alike (Cullen *et al.*, 1971), as is their thermotropic phase behaviour (Cronan *et al.*, 1979; Silvius and McElhaney, 1979). However, recent ^2H NMR studies indicate that the cyclopropane ring constitutes a barrier to propagation of motion along the acyl chain and is, at least in this sense,

distinct in its behavior from that of the *cis*-double bond (Dufourc *et al.*, 1983; Jarrell *et al.*, 1983; Dufourc *et al.*, 1984). These results support the hypothesis that cyclopropane fatty acids are associated with increased organizational stability in the membrane, while simultaneously permitting a degree of "fluidity" consistent with proper membrane functioning.

In order to more directly compare the properties of cyclopropane ring and monounsaturated substituted fatty acids in a lipid bilayer, *A. laidlawii* B membranes were enriched with *cis*- or *trans*-cyclopropyl fatty acids (19:0cp,c Δ 9, 19:0cp,t Δ 9) and *cis*- or *trans*-unsaturated fatty acids (18:1c Δ 9, 18:1t Δ 9) plus small amounts of one of the isomeric series of monofluoropalmitic acids. As previously, the thermotropic behavior of the membrane lipids was determined via DSC. Table 9 lists the fatty acid composition for each case of enrichment with either 18:1c Δ 9, 18:1t Δ 9, 19:0cp,c Δ 9 or 19:0cp,t Δ 9 plus a particular isomeric MFPA. In all cases the fatty acids provided exogenously accounted for greater than approximately 97% of the membrane lipid fatty acids. The products of *de novo* fatty acid biosynthesis in *A. laidlawii* B (12:0, 14:0, 16:0 and 18:0, Saito *et al.*, 1977) contributed minimally to the overall membrane lipid fatty acid composition, as expected for cells grown in the presence of avidin. In general, the ratio of the two fatty acids provided in the supplement was faithfully reflected in the membrane lipid fatty acid composition. However, some

Table 9: Fatty Acid Composition of A. laidlawii B Membrane Lipids Enriched with Various Isomeric MFPA's plus 18:1cΔ9, 18:1tΔ9, 19:0cp,cΔ9 or 19:0cp,tΔ9.

Supplement (0.12 mM Total)	Fatty Acid Composition (mole %)									
	12:0	14:0	16:0	18:1cΔ9	18:1tΔ9	19:0cp,cΔ9	19:0cp,tΔ9	19:0c	19:0t	NFPA
80% 18:1cΔ9 + 20%	---	---	1.9	72.8	---	---	---	---	---	29.3
+	---	---	2.8	73.2	---	---	---	---	---	24.0
+	---	---	2.2	75.9	---	---	---	---	---	21.9
+	---	---	1.9	71.3	---	---	---	---	---	26.8
+	---	---	2.0	73.7	---	---	---	---	---	24.3
80% 18:1tΔ9 + 20%	0.7	0.2	2.3	---	78.9	---	---	---	---	17.9
+	0.1	0.3	1.3	---	76.9	---	---	---	---	21.4
+	0.2	0.2	1.9	---	78.3	---	---	---	---	19.4
+	0.6	0.5	2.9	---	78.1	---	---	---	---	17.9
+	0.8	0.5	1.7	---	77.2	---	---	---	---	29.5
80% 19:0cp,cΔ9 + 20%	0.7	0.4	3.7	---	---	71.3	---	---	---	24.9
+	1.0	0.3	2.8	---	---	73.4	---	---	---	22.1
+	0.1	0.9	2.9	---	---	71.6	---	---	---	24.1
+	1.1	1.2	1.8	---	---	74.3	---	---	---	21.6
+	0.5	1.3	2.9	---	---	75.2	---	---	---	21.1
80% 19:0cp,tΔ9 + 20%	0.1	0.2	0.4	---	---	---	78.7	---	---	20.4
+	0.3	0.1	0.2	---	---	---	76.9	---	---	22.3
+	0.1	0.1	0.4	---	---	---	78.3	---	---	21.1
+	0.1	0.3	0.3	---	---	---	77.1	---	---	21.6
+	0.2	0.1	0.3	---	---	---	79.1	---	---	20.3

preference for the higher-melting MFPA's may be manifest in those cultures supplemented with the low-melting fatty acids 18:1c Δ 9 and 19:0cp,c Δ 9. This may be an attempt by the organism to compensate for the "hyperfluidizing" effect of these fatty acids on the membrane lipids.

Figure 28 illustrates endotherms obtained in the case of enrichment with 6F16:0 plus one of the particular fatty acids of interest. These endotherms are typical in that, for cases of enrichment with other isomeric MFPA's, the overall shapes of the endotherms were similar and the T_m (or T_m 's) varied by less than $\pm 1^\circ\text{C}$ about the average for that particular case. In the case of membrane lipids enriched with either 18:1c Δ 9 or 19:0cp,c Δ 9, a single, broad, somewhat asymmetric transition endotherm was observed. Most fatty acids, when incorporated into membranes of *A. laidlawii* B manifest this type of lipid phase transition endotherm (McElhaney, 1974). In contrast, the transition endotherms obtained with those membrane lipids enriched with 18:1t Δ 9 or 19:0cp,t Δ 9 were composed of two partially resolved transitions. Such phase separations were observed previously in *A. laidlawii* B membrane lipids enriched with 18:1t Δ 9 and seem to be attributable to lipid head group immiscibility. Clearly the *trans*-configuration of either the monounsaturated or cyclopropane ring substituent is associated with more complex phase behavior of *A. laidlawii* B membrane lipids, since both structures lead to observable phase separations. Generally, the miscibility of

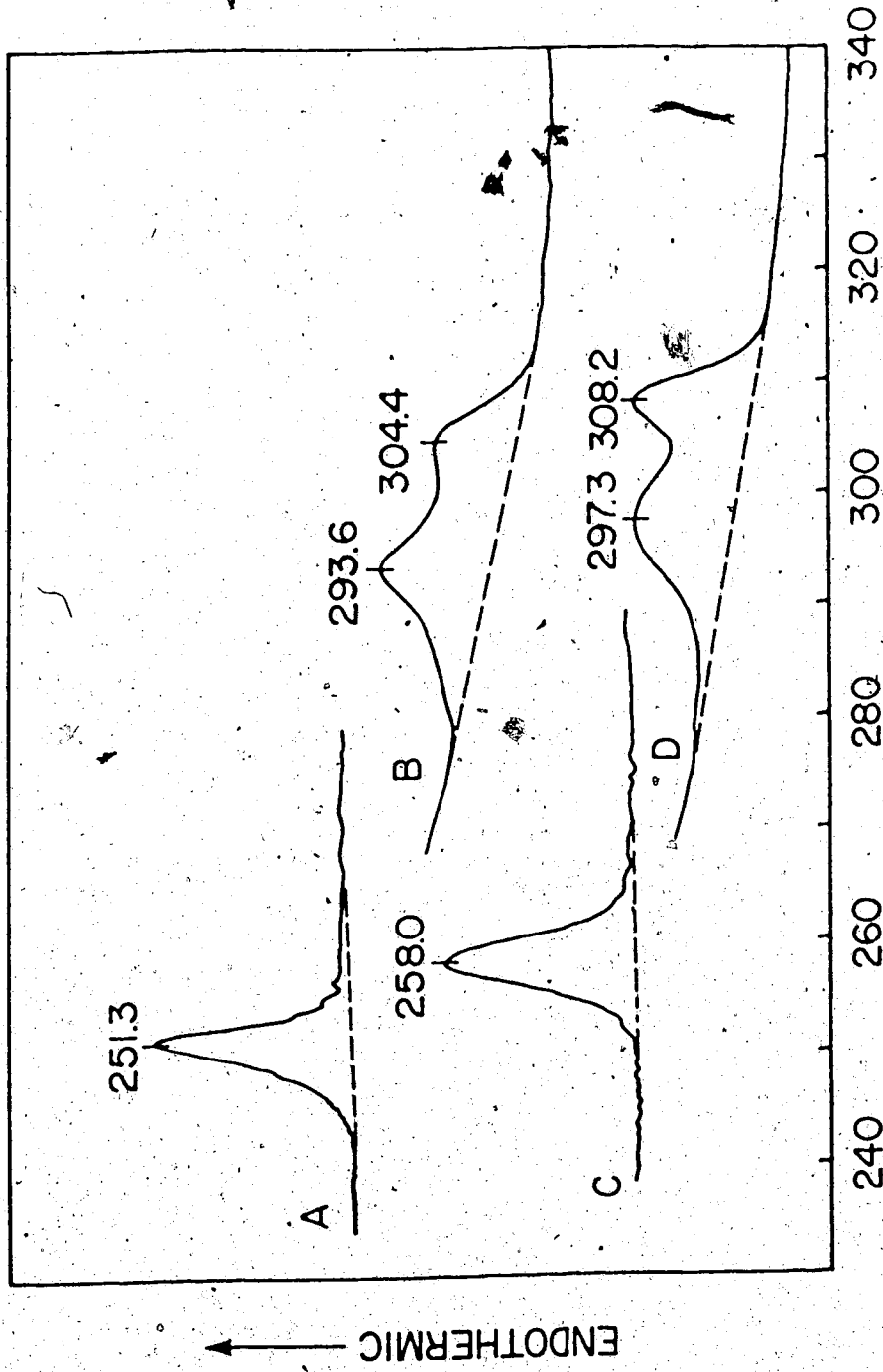


Figure 28: Lipid phase transition endotherms obtained by differential scanning calorimetry of the membrane polar lipid fraction from A. laidlawii B grown in the presence of 20 mole % 6F16:0 plus 80 mole % of A - 18:1cΔ9; B - 18:1cΔ9; C - 19:0cp,cΔ9; and D - 19:0cp,tΔ9. The scan rate was 5°C/min. The dashed line corresponds to the interpolated baseline.

the components of a mixture of lipids decreases as the difference between their phase transition temperatures increases (Mabrey and Sturtevant, 1976). In *A. laidlawii* B there exists the additional complication that lipid head group composition is altered under conditions of supplementation with different fatty acyl species (Silvius et al., 1980). Were it not for the fact that membrane lipids enriched with a very low-melting fatty acid (e.g., 18:1c Δ 9 or 19:0cp,c Δ 9) plus a relatively high-melting fatty acid (e.g., MFPA) showed no disposition towards phase separation, one might be tempted to invoke fatty acyl chain immiscibility as leading to the observed phase separation in membrane lipids enriched with 18:1t Δ 9 or 19:0cp,t Δ 9 plus MFPA. Since this explanation is untenable, and since similar phase separations are observed in membrane lipids which are 99.9% enriched with 18:1t Δ 9, it can only be suggested that the lipid head group composition of *A. laidlawii* B alters under conditions of enrichment with these *trans*-fatty acids in such a fashion as to lead to the calorimetrically observed phase separations. More detailed investigation into the interactions among the glyco- and phospholipid species of *A. laidlawii* B will be required before this point can be clarified.

Table 10 summarizes the calorimetric data for these various cases of enrichment. Briefly, the midpoint of the gel to liquid-crystalline phase transition (T_m) was always lowest when the structural substituent, either

Table 1: Calorimetrically Determined Gel to Liquid Crystalline Phase Transition Parameters for A. Laidlawii B Membrane Plus Lipid Enriched with Various Isomeric MFPA's Plus One of Either 18:1c:9, 18:1c:9, 19:0cp,c:9 or 19:0cp,c:9

Supplement (0.12mM Total)			Phase Transition Parameters ($^{\circ}\text{K}$)				
			T_1^{a}	T_2^{b}	$T_1 - T_2^{\text{c}}$	T_m^{d}	$\Delta T_{10-90}^{\text{e}}$
80% 18:1c:9	+ 20%	6F16:0	293.6	304.4	10.8	295.9	18.6
	+	8F16:0	293.1	301.2	8.1	295.4	17.6
	+	10F16:0	295.0	302.4	7.4	296.9	16.8
	+	12F16:0	293.9	302.1	8.2	296.0	16.4
	+	14F16:0	295.9	305.1	9.2	297.6	17.4
80% 18:1c:9	+ 20%	6F16:0	297.3	308.2	10.9	300.8	17.7
	+	8F16:0	296.4	307.3	10.9	300.4	17.8
	+	10F16:0	297.9	307.2	9.3	302.8	16.8
	+	12F16:0	299.1	309.6	10.5	302.4	16.7
	+	14F16:0	300.5	310.3	9.8	302.8	17.3
80% 18:1c:9	+ 20%	6F16:0	--	--	--	251.3	5.6
	+	8F16:0	--	--	--	253.6	4.8
	+	10F16:0	--	--	--	253.4	4.4
	+	12F16:0	--	--	--	253.3	4.3
	+	14F16:0	--	--	--	252.6	4.0
80% 19:0cp,c:9	+	6F16:0	--	--	--	258.9	6.0
	+	8F16:0	--	--	--	257.9	5.8
	+	10F16:0	--	--	--	257.7	6.1
	+	12F16:0	--	--	--	259.3	5.3
	+	14F16:0	--	--	--	258.4	5.7

^a T_1 corresponds to the maximum of the lower temperature transition.

^b T_2 corresponds to the maximum of the higher temperature transition.

^c $T_1 - T_2$ corresponds to the temperature separation of T_1 and T_2 .

^d T_m corresponds to the temperature at which the transition was 50% complete.

^e ΔT_{10-90} corresponds to the temperature range over which the transition passes from 10% to 90% of completion.

monounsaturated or cyclopropane ring assumed the *cis* configuration. The T_m 's of the two lipids with *cis* structural substituents (18:1c Δ 9, 19:0cp,c Δ 9) were very similar as were the T_m 's of the two lipids with *trans* structural substituents (18:1t Δ 9, 19:0cp,t Δ 9), although the lipids containing the cyclopropane ring substituent (either *cis* or *trans*) always melted several degrees higher than the lipids containing the monounsaturated substituents (either *cis* or *trans*). Values of ΔT_{10-90} were in the range 5°-10° for 18:1c Δ 9 and 19:0cp,c Δ 9, and of the order of 15°-20° for 18:1t Δ 9 and 19:0cp,t Δ 9, in good agreement with values of ΔT_{10-90} previously reported from this laboratory for *A. laidlawii* B membrane lipids (Silvius *et al.*, 1980).

The preceding calorimetric data were obtained with membrane polar lipid samples resuspended in ethylene glycol: water, 1:1 (v/v). While this has the advantage of eliminating the excess water transition which might otherwise obscure the lipid phase transition endotherm, it is possible that the presence of ethylene glycol might alter the thermotropic properties of the *A. laidlawii* B membrane lipids. For example, it has been known for some time that ethylene glycol can raise the observed T_m of phosphatidylcholines (PC's) containing unsaturated fatty acids but has little effect on the T_m of PC's containing saturated fatty acids (Van Dijk *et al.*, 1976). Figure 29 compares the DSC endotherms of *A. laidlawii* B membrane lipids enriched with either 18:1c Δ 9, 18:1t Δ 9 or 16:0 (grown in the absence of

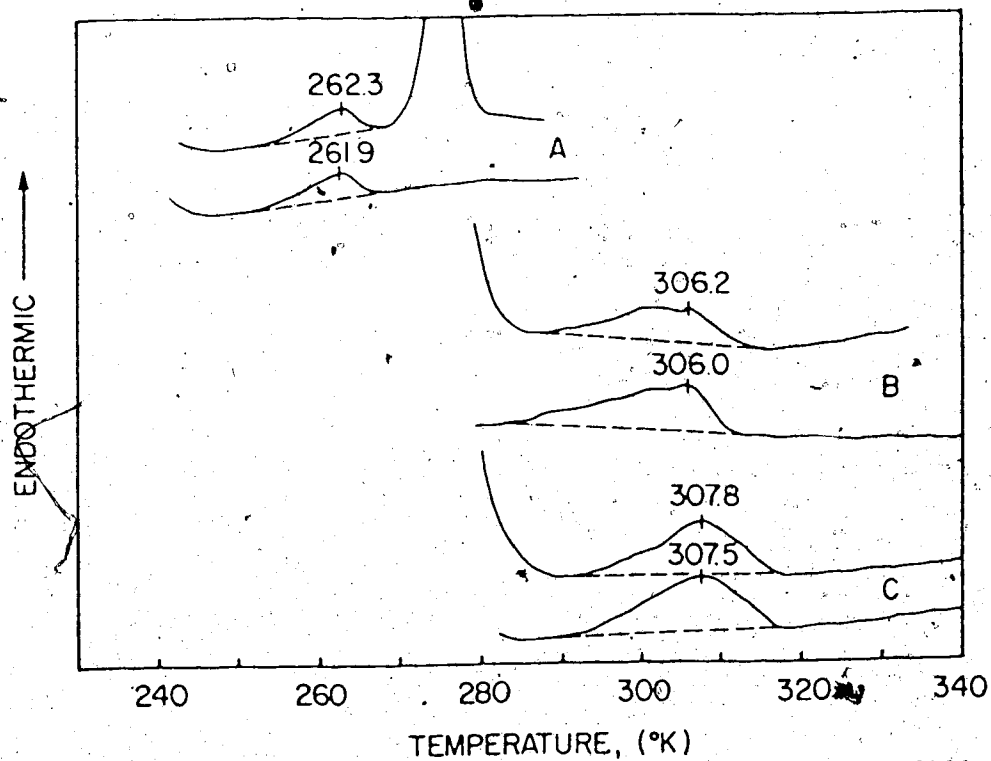


Figure 29: Lipid phase transition endotherms obtained by differential scanning calorimetry of the membrane polar lipid fraction from A. laidlawii B enriched with A - 18:1c Δ 9; B - 18:1t Δ 9; and C - 16:0. The lower endotherms were obtained in the presence of 50% ethylene glycol. The scan rate was 5°/min. The dashed line corresponds to the interpolated baseline.

avidin) and resuspended either in the presence or absence of ethylene glycol. Clearly, there are few differences in the thermotropic behaviour of these lipids attributable to ethylene glycol and certainly no systematic increase in T_m at lower phase transition temperatures in the presence of ethylene glycol.

Typical experimental ^{19}F -NMR spectra of 6F16:0 incorporated into the membrane lipids of *A. laidlawii* B in the presence of 19:0cp,t Δ 9 or 19:0cp,c Δ 9 are shown in Figure 30, along with the corresponding computer-generated simulated spectra. The values of the input parameters required to simulate the spectra are shown as well. These ^{19}F -NMR spectra were acquired over a range of temperatures which spanned the gel to liquid-crystalline phase transition of these membranes. The extensive broadening of the ^{19}F -NMR spectrum which occurs upon decreasing the temperature through the gel to liquid-crystalline phase transition is readily apparent, as is the fact that the spectra retain their essentially "superlorentzian" character even at temperatures well below the lipid phase transition. At all temperatures and in both the gel-state and the liquid-crystalline state, the ^{19}F -NMR spectra were adequately simulated using a model which assumes axially symmetric motions of the lipid molecules.

In the presence of 19:0cp,t Δ 9, over the range of temperatures tested, S_{m0} increased from approximately 0.19 at 320°K to 0.75 at 270°K. The value of Δ_0 remained constant

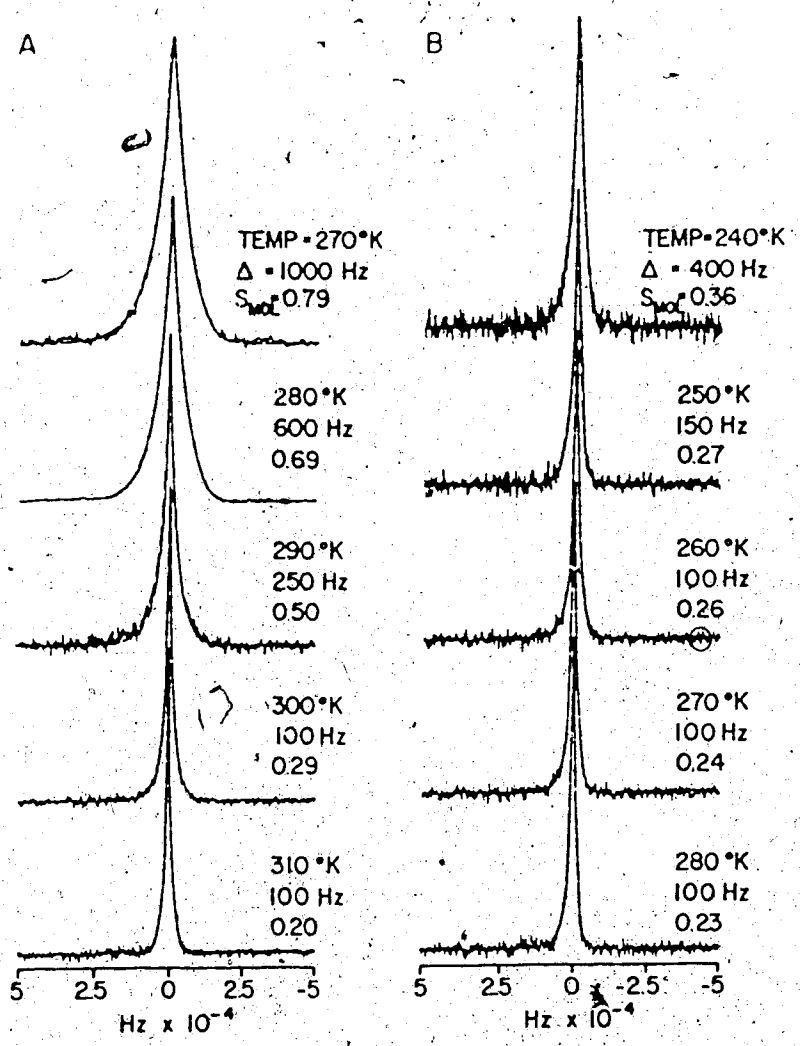


Figure 30: Experimental and simulated ^{19}F -NMR spectra of membranes of *A. laidlawii* B grown in the presence of 20 mole % 6F16:0 plus 80 mole % of A - 19:0cp,t Δ 9; and B - 19:0cp,c Δ 9. The simulated spectra are indicated in dashed lines superimposed upon the experimental spectra. The values of the computer input parameter Δ_0 (interchain dipolar broadening) and S_{mol} (molecular order parameter) are indicated for the simulated spectra.

at 100 Hz until the lipid phase transition was encountered and thereafter rapidly increased, reaching a value of 1000 Hz at 270°K. This is the same dependence of S_{m01} and Δ_0 upon temperature and membrane lipid phase state which is observed with most fatty acid-enriched *A. laidlawii* B membranes. In marked contrast to this situation was the dependence of S_{m01} on temperature and membrane lipid phase state in membranes of *A. laidlawii* B enriched with 19:0cp,c Δ 9. Here, S_{m01} increased from approximately 0.16 at 310° K to only 0.36 at 240° K, a temperature almost 20°C below the corresponding membrane lipid phase transition. Over the same temperature range, Δ_0 , the intermolecular interaction indicator, increased from 100 Hz to only 400 Hz. It does not in fact require a quantitative analysis of the ¹⁹F-NMR spectra to discern that the spectra obtained in the gel-state in the presence of 19:0cp,c Δ 9 reflect considerably greater motional averaging than the corresponding gel-state spectra obtained in the presence of 19:0cp,t Δ 9. This point may be ascertained qualitatively by a visual comparison of the spectra.

Figure 31 depicts the ¹⁹F-NMR order profiles of *A. laidlawii* B membranes enriched with either 18:1c Δ 9, 18:1t Δ 9, 19:0cp,c Δ 9 or 19:0cp,t Δ 9 obtained over a range of temperatures spanning the gel to liquid-crystalline phase transitions. In the liquid-crystalline state, in all cases of enrichment, the order profiles were very similar, showing a region of approximately constant orientational order

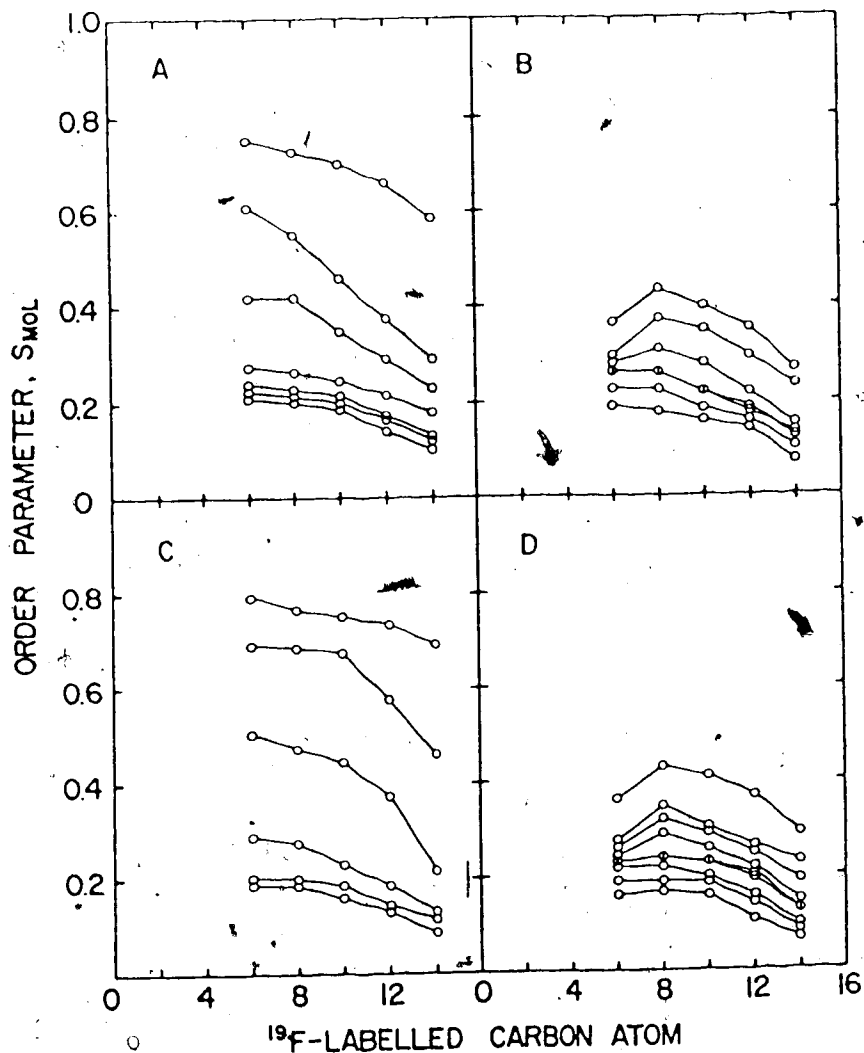


Figure 31: ^{19}F -NMR orientational order profiles of membranes of *A. laidlawii* B grown in the presence of 20 mole % various monofluoropalmitic acids plus 80 mole % of A - 18:1t Δ 9 (279, 284, 289, 294, 299, 304 and 309°K); B - 18:1c Δ 9 (240*, 250*, 266*, 279*, 279, 289 and 310°K); C - 19:0cp,t Δ 9; (270, 280, 290, 300, 310 and 320°K); or D - 19:0cp,c Δ 9 (240*, 250*, 260*, 270*, 280*, 280, 290, 300 and 310°K). The numbers in brackets represent the temperatures at which the order profiles, from top to bottom, were acquired. The asterisk represents the presence of 50% ethylene glycol.

preceding a decline in orientational order towards the methyl terminus of the acyl chain. Since the presence of a cyclopropane ring or a double bond on an acyl chain adjacent to the MPFA had little if any effect on the orientational ordering of the MFPA, it must be concluded that in the liquid-crystalline state the two fatty acyl chains experience a degree of independence sufficient to negate any influence of structural substituents in one acyl chain upon the orientational order of the other. Previous ^{19}F -NMR results have demonstrated that the orientational order profiles of the MFPA chains in membranes of *A. laidlawii* B are highly similar in the liquid-crystalline state whether those membranes were enriched with straight-chain saturated fatty acids such as 15:0 or 16:0 or any of an isomeric series of *cis*-octadecenoic acids or *trans*-octadecenoic acids or with methyl-branched fatty acids such as 16:0i and 16:0ai. ^2H -NMR results also generally indicate that the order profile is relatively invariant to alterations in fatty acyl structure in the liquid-crystalline state as discussed previously. The one exception to this generalization appears to be the cyclopropane ring where, even after correction for geometric considerations, the ^2H -NMR order profiles in both model (Dufourc *et al.*, 1983) and biological membranes (Jarrell *et al.*, 1983) indicate that the cyclopropyl substituent experiences a far greater degree of orientational ordering than any other portion of the fatty acyl chain. Nevertheless, when a deuterated palmitic acyl chain adjacent

to a cyclopropane ring containing acyl chain was monitored by $^2\text{H-NMR}$, the observed order profile gave little if any indication that the neighbouring cyclopropyl substituent had influenced the ordering of the palmitate chain (Dufourc *et al.*, 1984). It would appear then, from both $^{19}\text{F-}$ and $^2\text{H-NMR}$ results, that the elasticity of the fatty acyl chains in the liquid-crystalline state permits them to accommodate the presence of structural substituents without unduly altering the character of the orientational order profile.

With decreasing temperature, orientational order increased slowly and the character of the order profiles remained relatively constant until the lipid phase transition was encountered. As the proportion of gel-state lipid increased, overall orientational order increased markedly and the order profiles in the presence of different fatty acyl chain structures became increasingly dissimilar.

Consider first the cases of 18:1t Δ 9 and 19:0cp,t Δ 9. At a temperature of approximately 290°K, in both these enrichment situations, the membrane lipids have all but completely assumed the gel-state. Reflecting this condition, the values of S_{m01} at fluorine positions closer to the fatty acyl chain carbonyl head group indicated a pronounced increase in orientational ordering. Nevertheless, at fluorine positions near the fatty acyl methyl terminus, the increase in the value of S_{m01} was only relatively marginal. This disparity between the manner in which orientational order near the carbonyl head group and near the methyl

terminus altered with the increased proportion of gel-state lipid was then manifested as a pronounced head-to-tail gradient of orientational order in the presence of 18:1t Δ 9 or 19:0cp,t Δ 9. At temperatures sufficiently below the lipid gel to liquid-crystalline phase transition, the degree of orientational ordering was at all positions uniformly high, although a residual ordering gradient could still be discerned. Consequently the configuration of the MFPA chain in the gel-state in the presence of either 18:1t Δ 9 or 19:0cp,t Δ 9 may be described as overall highly ordered, approaching but not achieving an all-*trans* configuration with a residual head-to-tail gradient of orientational ordering. To put these observations in perspective, it should be noted that in the presence of straight-chain saturated fatty acids such as 15:0 or 16:0, the orientational order of the MFPA's increase approximately uniformly at all fluorine positions with increasing proportions of gel-state lipid and there remains little, if any, head-to-tail gradient of order in the gel-state. It would appear that the *trans*-cyclopropane ring or the *trans*-monounsaturated structural substituents were able to disrupt the gel-state packing of the fatty acyl chains, constituting a barrier to the assumption of a more highly ordered acyl chain configuration, which in turn is reflected in an order gradient in the gel-state.

Turning next to the cases of 18:1c Δ 9 and 19:0cp,c Δ 9, it can be seen that these structural substituents lead to

highly unusual gel-state orientational order profiles of the MFPA's. The orientational order at the six position in the presence of these substituents in the gel-state was always less than that observed at the eight position, suggesting that the MFPA chain is forced to bend around the bulky *cis*-cyclopropane ring or *cis*-double bond in the closely packed gel-state, or that these substituents are unable to pack closely in the gel-state, thus permitting the MFPA chain a greater degree of motional freedom in their immediate locale. Since the MFPA chain should preferentially distribute to the *sn*-1 position of the glycerolipid backbone in the presence of 18:1 Δ 9 or 19:0cp,c Δ 9 (by analogy with 16:0, Saito *et al.*, 1977), and given the physical inequivalence of the fatty acyl chains esterified to the *sn*-1 and the *sn*-2 position, it is not surprising that the greatest effect of a structural substituent located at the Δ 9 position on the *sn*-2 chain should be manifest at the C-6 position on the *sn*-1 chain. Previous $^1\text{F-NMR}$ results have indicated that a *cis*-double bond located near the methyl terminus of the fatty acyl chain can locally disrupt gel-state chain packing (as do methyl *iso*- and *anteiso*-branch structures), but that gel-state chain packing densities were sufficient to overcome any local disruptive effect of a *cis*-double bond located near the carbonyl head group of the fatty acyl chain. The present results indicate that structural substituents located near the center of the acyl chain are also capable of disrupting gel-state chain packing and indeed,

when the overall ordering achieved in the presence of such structures is considered, this would appear to be the most efficient position from which to influence gel-state ordering. Overall, the degree of ordering achieved in the presence of 18:1cΔ9 or 19:0cp,cΔ9 was much lower than that obtained in the presence of 18:1tΔ9 or 19:0cp,tΔ9. Thus the configuration of the MFPA chain in the gel-state in the presence of 18:1cΔ9 or 19:0cp,cΔ9 may be described as highly disordered, apparently reflecting both local and overall disruption of the density of gel-state fatty acyl chain packing.

The ¹⁹F-NMR spectra of *A. laidlawii* B membranes enriched with either 18:1cΔ9 or 19:0cp,cΔ9 were acquired in the presence of 50% ethylene glycol at temperatures of 270°K or lower in order to prevent sample freezing. It has already been noted that the presence of 50% ethylene glycol had little if any effect on the thermotropic properties of *A. laidlawii* B membrane lipids, whether those lipids were enriched with 18:1cΔ9, 18:1tΔ9 or 16:0. It seems unlikely then that the presence of ethylene glycol unduly influenced the position of the membrane lipid phase transition during acquisition of the ¹⁹F-NMR spectra at temperatures below 270°K. The ¹⁹F-NMR spectra of *A. laidlawii* B membranes enriched with 20% 6F16:0 plus 80% 18:1tΔ9 at 279°K were identical whether acquired in the presence or absence of 50% ethylene glycol, so that ethylene glycol itself did not prevent the membrane lipid fatty acyl chains from assuming a

highly ordered, nearly all *trans*-configuration (data not shown). The ^{19}F -NMR order profiles of *A. laidlawii* B membranes enriched with either 18:1c Δ 9 (or 19:0cp,c Δ 9) were obtained at 279°K (or 280°K) in the presence or absence of 50% ethylene glycol. As shown in Figure 31, the order profiles at this temperature were essentially identical whether obtained with or without ethylene glycol. These various observations strongly suggest that the highly disordered gel-state assumed in the presence of 18:1c Δ 9 or 19:0cp,c Δ 9 is unrelated to any effect of ethylene glycol, but rather is characteristic of the influence of the *cis*-cyclopropane ring and the *cis*-double bond upon the motional freedom of the MFPA probe in the gel-state.

Further insights into the consequences of the inclusion of *cis*- or *trans*-cyclopropyl ring or monounsaturated substituents can be gained by a direct comparison of the overall orientational order of the MFPA probes at a particular temperature in the presence of a particular structural substituent. The chain-average order parameters are plotted versus the acquisition temperature in Figure 32 for the four cases of enrichment with either 18:1c Δ 9, 19:0cp,c Δ 9, 18:1t Δ 9 or 19:0cp,t Δ 9. Several significant features become evident from Figure 32. At 310°K, a temperature above the lipid phase transition of all four types of enriched membranes, overall orientational order was generally very similar regardless of the particular fatty acyl chain structure examined. For any one acquisition temperature at which all

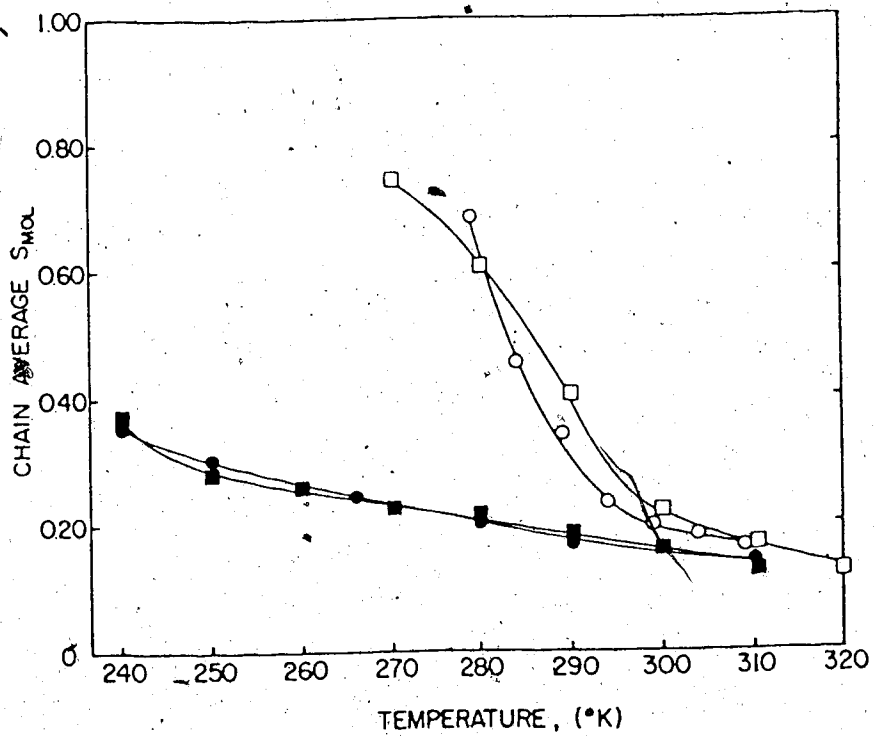


Figure 32: Chain average orientational order versus acquisition temperature for enrichment with cis and trans-cyclopropane ring and unsaturated fatty acids. The chain average order was calculated as described in the text. Open circles, 18:1tΔ9; open squares, 19:0cp,tΔ9; closed circles, 18:1cΔ9; closed squares, 19:0cp,cΔ9.

fatty acyl chain structures are in the liquid-crystalline state, overall order is relatively independent of specific acyl chain structure and is an approximately inverse linear function of $(T - T_m)$ (i.e., order decreases as proximity to the lipid phase transition decreases, or as $T - T_m$ increases). In keeping with this observation, it can be discerned that at 310°K the overall order in the presence of 18:1cΔ9 or 19:0cp,cΔ9 was approximately 0.14 ($[T - T_m] \cong 50^\circ\text{C}$), while in the presence of 18:1tΔ9 or 19:0cp,tΔ9 overall order was approximately 0.17 ($[T - T_m] \cong 10^\circ\text{C}$). This effect is of course quite small and at sufficiently high temperatures overall order should become independent of $(T - T_m)$.

As the acquisition temperature was lowered, overall orientational order increased in a linear fashion until the membrane lipid gel to liquid-crystalline phase transition was encountered. In the case of membranes enriched with 18:1tΔ9 or 19:0cp,tΔ9, between 300°K and 290°K the overall order began to increase markedly, accurately reflecting the position of the calorimetrically determined lipid phase transition. Nevertheless, even at temperatures below the lipid phase transition, the fatty acyl chains evinced substantial disordering and only at temperatures well below the phase transition was the highly ordered, nearly all-*trans* conformation approached. In addition, it is evident that the cyclopropane ring and the double bond, when in the *trans* configuration, behaved more or less similarly

with respect to the lipid phase transition in terms of their effect on the overall ordering of the MFPA probes. These same points can be made concerning the cyclopropane ring and the double bond when in the *CIS* configuration. The overall order of the MFPA probes increased in an approximately linear fashion with decreasing temperature in the liquid-crystalline state and the two structural substituents affected the orientational order of the MFPA probes approximately equally. Nevertheless, it can be discerned that the MFPA's in the gel-state in the presence of these structural substituents when in the *CIS* configuration did not achieve the same degree of overall ordering as when in the *trans* configuration.

Figure 33 depicts the chain-average order parameter as a function of the reduced temperature, T_r , for each case of enrichment with 18:1c Δ 9, 19:0cp,c Δ 9, 18:1t Δ 9 or 19:0cp,t Δ 9. In the presence of the two fatty acids 18:1t Δ 9 and 19:0cp,t Δ 9, the overall order of the MFPA's showed a profound increase at the temperature of the lipid phase transition ($T_r = 0$), indicating again that the lipid phase transition was the preeminent affector of overall orientational order. Both fatty acids again behaved more or less similarly with respect to T_r although significant differences in the overall order between 18:1t Δ 9 and 19:0cp,t Δ 9 were evident at values of $T_r < -0.05$. Whether these disparities reflect real differences in the response of the *trans*-cyclopropane ring and the *trans*-double bond to

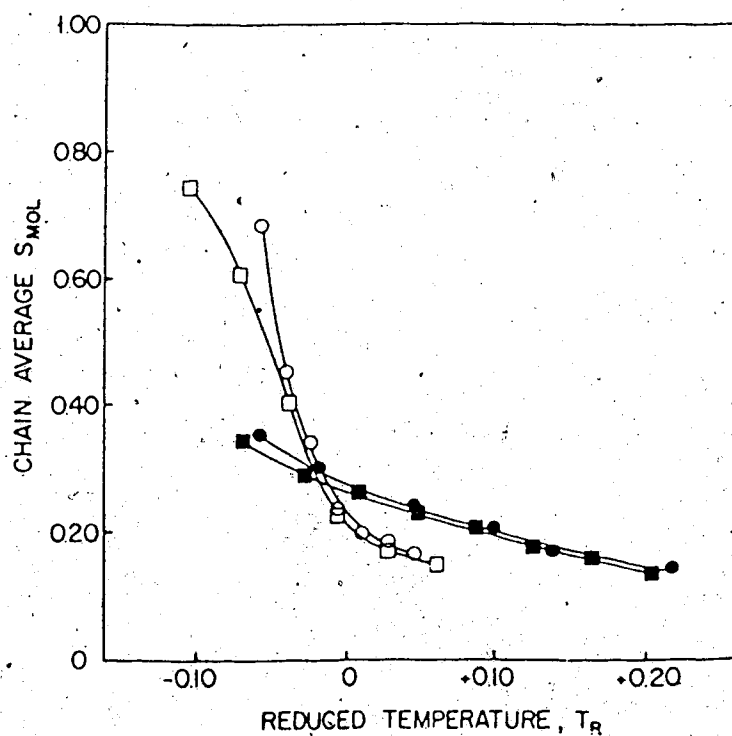


Figure 33: Chain average orientational order versus reduced temperature for enrichment with cis and trans-cyclopropane ring and unsaturated fatty acids. The chain average order and T_R were calculated as described in the text. Open circles, 18:1t Δ 9; open squares, 19:0cp,t Δ 9; closed circles, 18:1c Δ 9; closed squares, 19:0cp,c Δ 9.

the gel-state or whether they result from differences in the distribution of the MFPA's between the two calorimetrically observed phase transitions in the presence of these *trans* structures cannot be unequivocally stated. However, in a previous $^1\text{F-NMR}$ study of an isomeric series of *trans*-octadecenoic acids, values of the chain-average order in the presence of 18:1t Δ 6 or 18:1t Δ 11 approached 0.75 at a value of $T_r \cong -0.10$, indicating that the differences between 19:0cp,t Δ 9 and 18:1t Δ 9 in the gel-state may be more apparent than real.

It is clear from Figure 33 that in the presence of the fatty acids 18:1c Δ 9 and 19:0cp,c Δ 9, the MFPA's did not show the sharp increase in overall order upon encountering the lipid phase transition that was evident in the presence of 18:1t Δ 9 and 19:0cp,t Δ 9, and that the overall order achieved in the gel-state, at values of T_r where the *trans* acids were approaching an all-*trans* configuration, indicates that the *cis* acids remained highly disordered. Evidently these structures when in the *cis*-configuration are capable of disrupting gel-state acyl chain packing in both a local and overall sense, effectively preventing or acting as a barrier to the ready assumption of an all-*trans* configuration of the acyl chains in the gel-state. If the temperature of the membrane lipid gel to liquid-crystalline phase transition is viewed as a measure of the stability of the gel-state, then gel-state disordering or instability leads to a lower phase transition temperature. The implication is then that

specific fatty acyl structural substituents alter the stability of the gel-state through disruption of gel-state acyl chain packing and hence lower the lipid phase transition temperature. The same interpretation has been used to rationalize the relative overall gel-state ordering of straight-chain saturated versus methyl *iso*- and *anteiso*-branched fatty acids and their respective membrane lipid phase transition temperatures.

It is further evident from Figure 33 that in the liquid-crystalline state (i.e., $T_r > 0$), at comparable values of T_r , those membranes enriched with the lower-melting fatty acids 18:1c Δ 9 or 19:0cp,c Δ 9 were more highly ordered than those enriched with the higher-melting fatty acids 18:1t Δ 9 or 19:0cp,t Δ 9. This same relationship, that is, the lower the lipid phase transition the higher the overall order at equal values of $T_r > 0$, has been observed using ^{19}F -NMR in *A. laidlawii* B membranes enriched with a multitude of various fatty acyl structures. Liquid-crystalline state packing considerations seem insufficient to explain this relationship, since monolayer studies indicate that most fatty acyl structural types occupy approximately equal cross-sectional areas in the liquid-expanded state (see, for example, Kannenburg *et al.*, 1983). In the absence of a phase change, order increases in a more or less linear fashion with decreasing temperature, as can be ascertained from Figure 32. When it is noted that at a value of $T_r = +0.05$ one is comparing the order of 18:1t Δ 9

(or 19:0cp,t Δ 6) at approximately 310°K with the order of 18:1c Δ 9 (or 19:0cp,t Δ 9) at approximately 270°K, it can be taken that the dependence of order on absolute temperature in the liquid-crystalline state is sufficient to account for the observed differences.

An Overview of the Relationship Between Fatty Acid Structure, Orientational Order, and the Lipid Phase Transition

In this final section, I would like to extract from the order parameter data whatever generalities exist concerning the relationship between fatty acid structure, orientational order and the temperature of the gel to liquid-crystalline phase transition.

The immediate consequence of altering fatty acyl chain structure is that the temperature of the gel to liquid-crystalline phase transition is altered. Are the alterations in T_m which occur in model systems, such as PC multilayers, upon substituting particular fatty acid structures, faithfully reflected in the more complex biological system of *A. laidlawii* B membranes? Figure 34 compares directly the T_m reported herein for membrane lipids of *A. laidlawii* B made homogeneous with respect to particular fatty acids and the T_m reported by various laboratories for PC model membranes containing the same particular fatty acids. While the correlation between the two sets of data is good, it is not exact. Linear regression analysis indicates that the

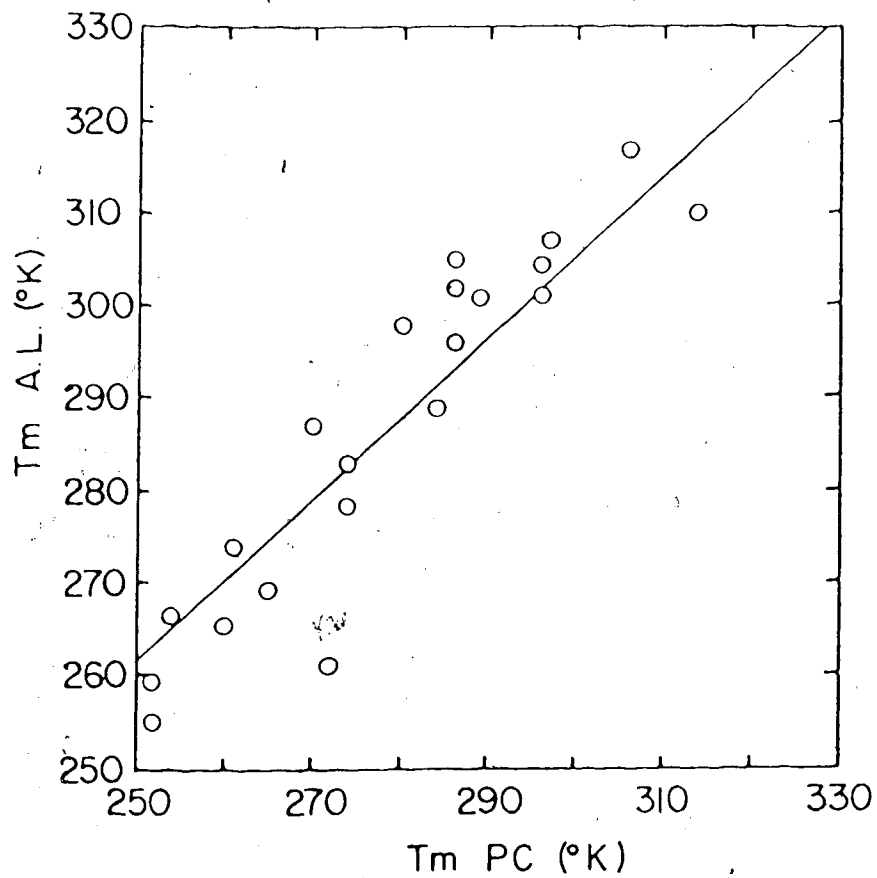


Figure 34: Correlation of T_m (*A. laiclavii* B) with T_m (PC). The T_m (PC) data were taken from Silvius (1982). The correlation function fits a line with slope of 0.875 with a linear correlation coefficient of $r=0.93$.

correlated data fit a line with a slope equalling 0.875. Thus the *A. laidlawii* B membrane lipids generally experience an order-disorder transition at a higher temperature than the corresponding PC, and the differential becomes more pronounced at lower values of T_m . This differential cannot be entirely attributed to the presence in the *A. laidlawii* B membrane lipids of 10 to 20 mole % of the generally higher melting MFPA. A similar correlation analysis performed by Silvius (1979) indicated that the correlated data fit a line with slope equalling 0.91. Therefore the same characteristics are displayed by *A. laidlawii* B "homogeneous" membranes containing no MFPA. It may well be that *A. laidlawii* B membrane lipids are intrinsically higher melting than PC. A study by Silvius *et al.* (1980), in which the *A. laidlawii* B membrane lipid headgroup species were isolated and their individual thermotropic properties characterized, demonstrated that the two neutral glycolipids MGDG and DGDG displayed phase transition endotherms 10-15°C higher than the major phospholipid species of *A. laidlawii* B, PG, which behaved similarly to PC's. The important conclusion is, nevertheless, that the effects of particular fatty acyl structures upon the thermotropic properties of synthetic systems are reflected in *A. laidlawii* B membrane lipids.

It has been demonstrated in the present studies that in the liquid-crystalline state the shape of the fatty acyl chain order profile of the MFPA's is relatively invariant to

changes in the particular fatty acyl structure with which the *A. laidlawii* B membrane lipids are coenriched. The hydrocarbon chains of the lipid bilayer in its fluid state are permitted a wider range of motional freedom and, on the average, occupy far greater cross-sectional areas than characteristic of their condition in the gel-state. It follows that individual hydrocarbon chains experience weaker interactions with their neighbours, that they become to a degree more independent of any influence of one chain upon another, and that the introduction of structural substituents should little affect the condition of an adjacent hydrocarbon chain. Statistical-mechanical models of hydrocarbon chain motions and conformations in a lipid bilayer successfully exploit this situation and treat interactions between chains, including chains esterified to the same glycerol backbone, as a "mean field" interaction composed of average repulsive (steric) and attractive (van der Waals) forces (Marcelja, 1974; Meraldi and Schlitter, 1981a, b; Dill and Flory, 1980). Does it therefore follow that the S_{m0} orientational order parameters can be characterized in terms of "average" forces in the liquid-crystalline state, independent of the details of particular structural substituents and, what are those forces?

For each of the 21 different fatty acyl chain structures studied, the value of S_{m0} was determined at both 279°K and 310°K. In Figure 35 the value of the chain-average S_{m0} obtained at either 279° or 310°K for a particular case

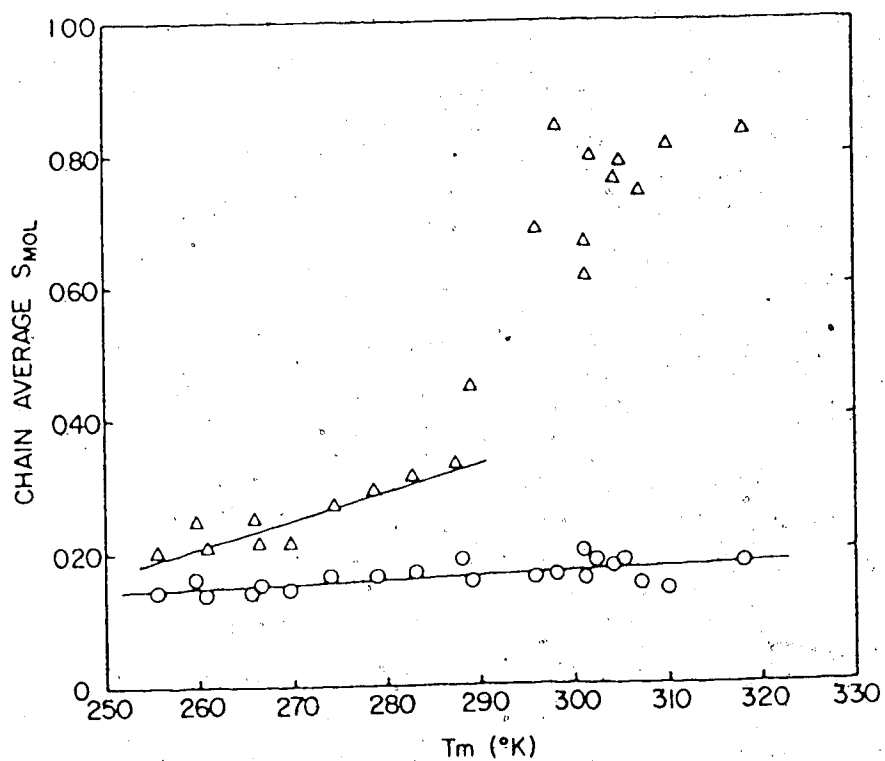


Figure 35: Chain average orientational order at a constant temperature versus T_m for each fatty acyl structure. The data at 310°K (circles) fit a line with slope= 7×10^{-4} , y intercept= -0.03 with a linear correlation coefficient of $r=0.77$. The data at 279°K (triangles) fit a line with slope 32×10^{-4} , y intercept -0.63 with a linear correlation coefficient of $r=0.60$.

of enrichment is plotted versus the corresponding T_m of the particular *A. laidlawii* B membrane lipids. At 310°K all data points fall more or less on a straight line with a slope near zero. At this temperature, in all but one of the cases of enrichment, the membrane lipids have assumed the liquid-crystalline state. Therefore at 310°K overall order is practically independent of the particular fatty acyl structure, and proximity to the phase transition has only a minimal influence on relative order. At 279°K about half of the 21 different fatty acyl chain structures studied were in the liquid-crystalline state and the remainder had assumed the gel-state. Those structures which retained the liquid-crystalline state at 279°K displayed the same linear relationship between overall order and T_m , again apparently independent of the particular fatty acyl structure, as was observed at 310°K, although the slope of the line was more positive. This observation suggests that proximity to the T_m has a greater influence upon relative overall order at lower temperatures. When the acquisition temperature was below the T_m of the membrane lipids, overall order increased rapidly and relative order became increasingly dependent upon the particular fatty acyl structure.

The effect of proximity to the T_m upon overall orientational order is more directly obtained when the chain-average S_{m0} , for a particular case of enrichment is plotted as a function of $(T - T_m)$ as in Figure 36, where T is the acquisition temperature. Again, at 310°K, overall order

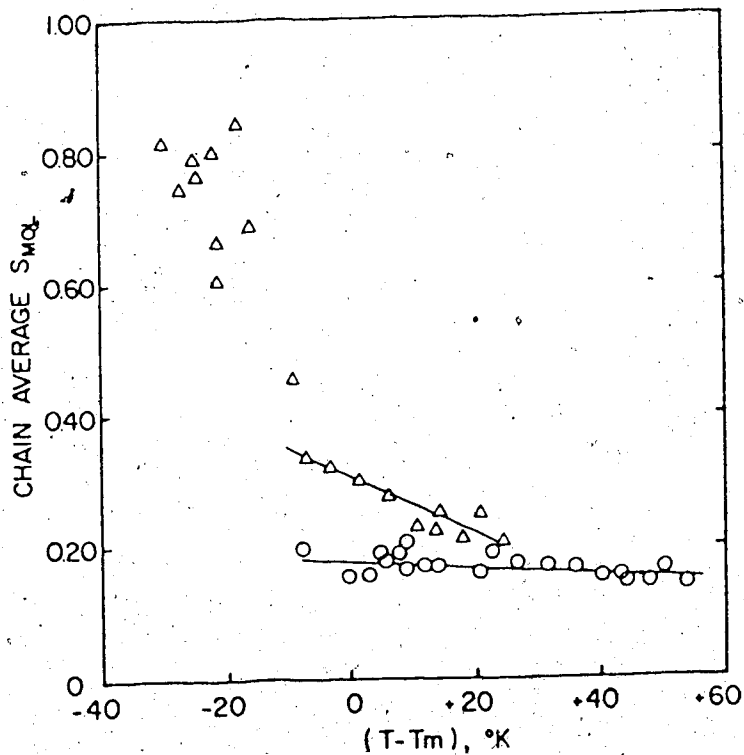
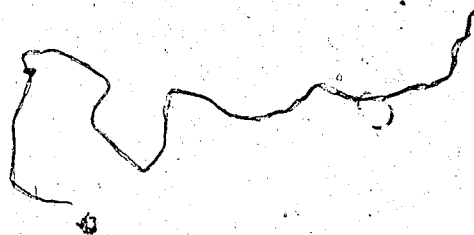


Figure 36: Chain average orientational order at a constant temperature versus $(T-T_m)$ for each fatty acyl structure. The data at 310°K (circles) fit a line with slope= -9×10^{-4} , y intercept=0.19 with a linear correlation coefficient of $r=0.82$. The data at 279°K (triangles) fit a line with slope= -44×10^{-4} , y intercept=0.30 with a linear correlation coefficient of $r=0.81$.



was an approximately linear function of $(T-T_m)$, apparently independent of the particular fatty acyl structure. At 279°K, provided $(T-T_m) \geq 0$, overall order was linearly related to the proximity to T_m and the dependence was again more pronounced than at 310°K. Below $(T-T_m) \cong 0$, order increased rapidly as the proportion of gel-state lipid increased.

The presentations of the data in Figures 35 and 36 suggest that, in the liquid-crystalline state, orientational order at any one temperature is relatively independent of fatty acyl structural substituents and depends more on "average" forces such as proximity to the lipid phase transition. Furthermore, the strength of this dependence is a function of temperature. Therefore, the following equations describe the relationship between ordering, S , and T_m and $(T-T_m)$,

$$S = mT_m + b \quad [1]$$

$$S = n(T-T_m) + c \quad [2]$$

provided $T \geq T_m$. The slopes m and n , and the intercepts b and c , are functions of the acquisition temperature T , and can be estimated by linear regression analysis of the data at 279°K and 310°K. Further values of the slope and intercepts were obtained for intermediate temperatures by analysis of the order data for various fatty acyl structures

obtained at 290, 300 and 320°K. The dependence of the slopes m and n , and of the intercepts b and c , upon the acquisition temperature T are illustrated in Figure 37 for the situation $T \geq T_m$. To a good approximation all four quantities are linear functions of temperature. At sufficiently high temperatures the overall order is no longer a function of T_m or $T - T_m$ since the slopes tend toward zero. In the case of *A. laidlawii* B membrane lipids, this occurs at about 320°K. Above this temperature overall order should be simply a direct function of temperature, independent of T_m or $T - T_m$. Thus the merger of the values of the intercepts b and c at 320°K reflect this independence. The temperature dependence of the four quantities in the temperature range 280°K to 320°K may be expressed approximately as linear functions and these are:

$$m = -8.5 \times 10^{-5} \cdot T + 0.027 \quad [3]$$

$$n = +12.0 \times 10^{-5} \cdot T - 0.038 \quad [4]$$

$$b = 0.0191 \cdot T - 5.958 \quad [5]$$

$$c = -0.00357 \cdot T + 1.298 \quad [6]$$

where m and b , and n and c , are the slope and y intercept of the dependence of S_{m0} on T_m and $(T - T_m)$, respectively, while T is the acquisition temperature in °K. Provided that $T \geq T_m$, the order parameter data of Figures 35 and 36 may be predicted approximately for any fatty acid structure using the above equations and knowledge of T_m and T .

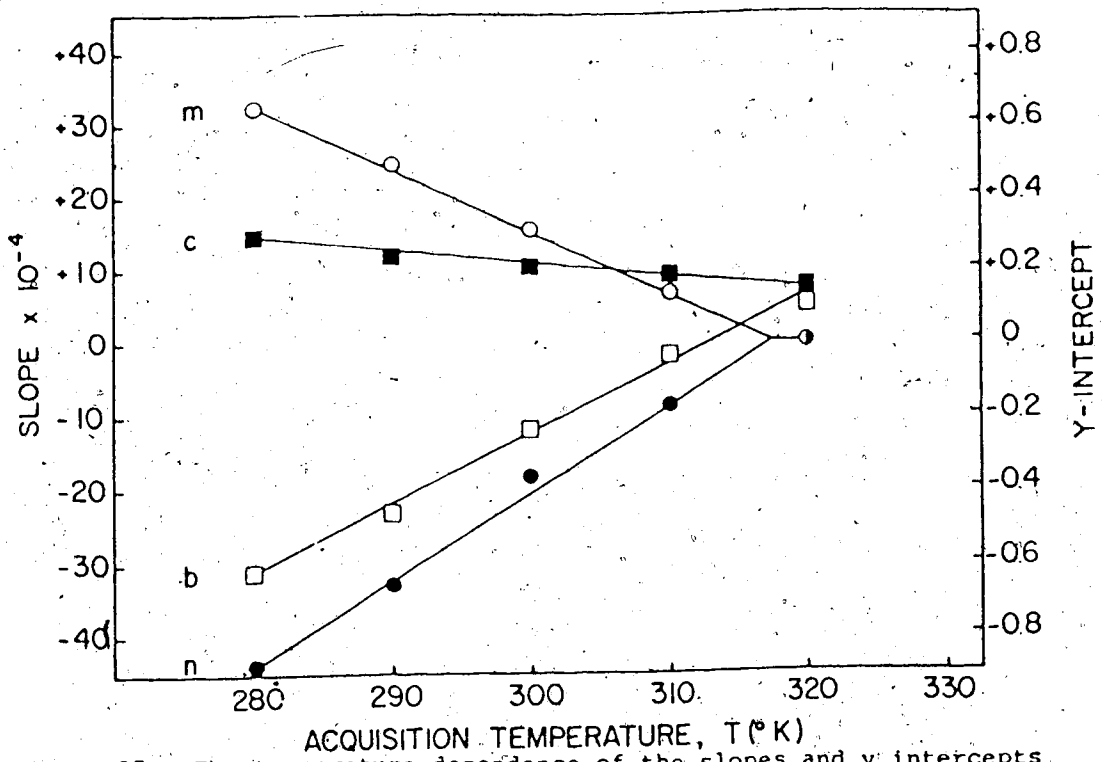


Figure 37: The temperature dependence of the slopes and y intercepts of the lines describing the relationship between order and T_m or $(T-T_m)$ at a constant temperature. Slope and y intercept (open circles, open squares) for order versus T_m . Slope and y intercept (closed circles, closed squares) for order versus $(T-T_m)$.

The success of the preceding treatment of the orientational order in the liquid-crystalline state as simple functions of temperature and proximity to the phase transition, essentially independent of the structure of the particular fatty acid, cannot be translated to the gel-state. Both the shapes of the order profiles and the relative overall ordering in the gel-state vary widely in the presence of different fatty acyl structural substituents. This point is most effectively portrayed in Figure 38, where the chain average S_{m0} is plotted as a function of the reduced temperature, T_r for different fatty acid structures. Included are representatives of the linear saturated fatty acids, *cis*- and *trans*-monounsaturated and methyl *iso*- and *anteiso*-branched fatty acids. The *cis*- and *trans*-cyclopropane ring-containing fatty acids are not shown for reasons of clarity, since their behaviour with respect to T_r has already been shown to be nearly identical with their monounsaturated homologues. Thus Figure 38 contains representations of the effect of the lipid phase transition on the orientational order of the MFPA's in the presence of fatty acyl species from each of the naturally occurring classes of fatty acid. It is evident that at an equivalent value of the reduced temperature below the normalized phase transition (e.g., $T_r = -0.10$), different fatty acid structures assume markedly different degrees of overall orientational ordering. Figure 39 illustrates the dependence of overall ordering at a constant value of $T_r \leq 0$ upon the

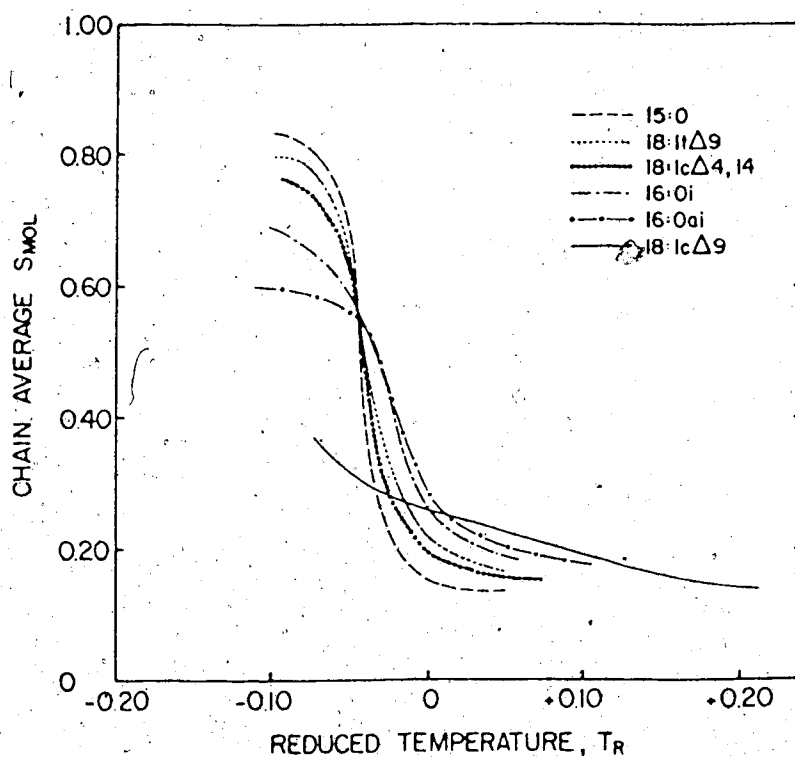


Figure 38: Normalization of the chain average order parameters with respect to the lipid phase transition for each class of fatty acid structure studied. 15:0 (---), 16:0i (-·-·-), 16:0ai (—), 18:1t Δ 9 (·····), 18:1c Δ 4,14 (○○○○○), 18:1c Δ 9 (—). The cis and trans-cyclopropane ring fatty acids are excluded for reasons of clarity.

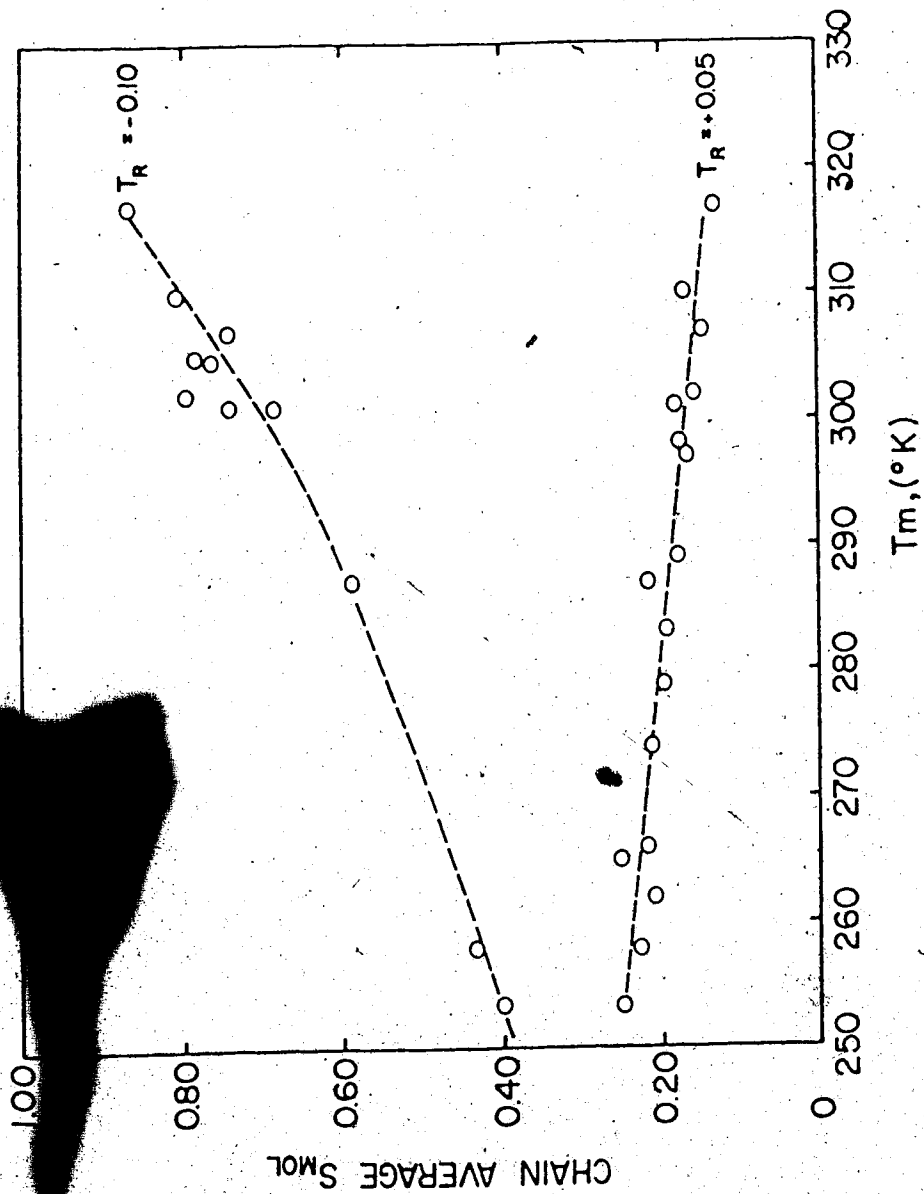


Figure 39: Chain average order at a constant value of reduced temperature above or below the lipid phase transition versus the temperature of the lipid phase transition.

T_m of the corresponding fatty acid-enriched *A. laidlawii* B membrane lipids. As the T_m decreased, overall orientational order decreased. This observation suggests that different fatty acyl structures exert their influence upon the temperatures of the phase transition by affecting the stability of gel-state chain packing. Of course, this is not a new concept. Monolayer studies have long indicated that, at equivalent surface pressures, lipids containing such fatty acyl structural substituents as *cis*- and *trans*-double bonds or cyclopropane rings or methyl branches occupy larger cross-sectional areas in the liquid-condensed state (Chapman *et al.*, 1966; Kannenburg *et al.*, 1983). X-ray diffraction studies have demonstrated that specific structural substituents such as the methyl *anteiso*-branch can affect gel-state packing (Bouvier *et al.*, 1981). In fact, simply examining molecular models of various fatty acyl structures demonstrates the difficulty of closely packing such structures as cyclopropane ring-containing fatty acids.

In Figure 39 the overall orientational order as a function of T_m is also plotted at a constant value of $T_r \geq 0$ (i.e., in the liquid-crystalline state). In contrast to the situation at $T_r \leq 0$, it is observed that overall order decreases as a function of increasing T_m . Since fatty acyl chain structure and hence chain-packing considerations seem to have little bearing on overall order in the liquid-crystalline state, the origin of this effect is not immediately obvious. Three simple equations are pertinent:

$$S = mT_m + b \quad [1]$$

$$S = n(T - T_m) + c \quad [2]$$

add,

$$\theta = (T - T_m) / T_m = T_r \quad [7]$$

By straight-forward algebraic manipulation, the following equation is found to relate the order, S , and the reduced temperature, T_r :

$$S = b - \left[\frac{\frac{m}{n} (b - c)}{\frac{m}{n} - T_r} \right] \quad [8]$$

Since m , n , b and c can be expressed in terms of temperature alone, this equation describes the temperature dependence of the orientational order at any one value of $T_r \geq 0$.

Substituting for the appropriate values of m , n , b and c , this relation predicts that at $T_r = +0.05$, the ratio of the values of S at 310°K and 280°K should equal:

$$\frac{S_{310}}{S_{280}} = 0.743$$

The actual value of S_{310}/S_{280} obtained from Figure 39 is 0.75. This implies that the slope of the S versus T_m data at a constant T_r of +0.05 can be attributed entirely to the effect of temperature on order in the liquid-crystalline state and that it is unnecessary to invoke any peculiar property of the fatty acid in the liquid-crystalline state. The reason that a more ordered state is observed for those

membrane' lipids undergoing a phase transition at lower temperatures is that they are then in a liquid-crystalline state at a lower temperature.

The data in Figure 39 suggest a further consequence of destabilizing the gel-state. If the disruptive influence of a particular structural substituent is great enough there will be no observable phase transition in the lipid bilayer. At some temperatures the differences in the S versus T_m dependencies at $T_r = -0.10$ and $T_r = +0.05$ will vanish and there would then be no difference in ordering between the two states and by definition no phase transition.

Consider an isolated acyl chain in an all-*trans* configuration. As the temperature begins to increase, first one and then another methylene segment is excited to undergo *trans/gauche* isomerization. There is nothing abrupt about the increase in the probability of rotational isomerization. It increases more or less progressively with increasing temperature. Now consider the same acyl chain packed amongst its neighbours in the gel phase of a lipid bilayer. Were a particular methylene segment to rotate into a *gauche* \pm conformation, it would "bump" into a neighbouring chain. In order for the rotational isomerization to occur, the methylene segment on the neighbouring chain must undergo a simultaneous rotational isomerization. It is this necessity for cooperative or concerted conformational change that leads to the abruptness of observed lipid phase transitions. When these essentially steric restrictions are relieved,

when the fatty acyl chains do not interact so closely in the gel-state, rotational isomerizations occur more readily and therefore at a lower temperature. In extreme cases there is no calorimetrically observable phase transition.

In attempting to model the relationship between chain interactions and the temperature of the lipid phase transition, it might first be assumed that the thermal energy required to destabilize gel-state chain packing will be proportional to the minimum interaction energy (E_0) of the chains, as defined by Shapiro and Ohki (1974). These workers calculated theoretical values of E_0 for hydrocarbon chains of various lengths from 2 to 18 carbons in an all-*trans* configuration with a constant interchain distance, d . Secondly, it will be assumed that, to a reasonable approximation, the total interaction energy for a chain containing some structural substituent such as a double-bond, a methyl branch or a cyclopropane ring, can be estimated as the sum of the interaction energies of the two constituent all-*trans* segments. For example, E_0 for 18:1c Δ 7 chains would be equal to the sum of E_0 for a heptane segment plus E_0 for an undecane segment. Implicit within this second assumption is a third; that the presence of a structural substituent will *not* alter the interchain spacing, d . The model described above is essentially that proposed by Barton and Gunstone (1975).

In order to test this model, the minimum interaction energies were calculated for various types of fatty acyl

chains using the data of Shapiro and Ohki (1974) and plotted versus the corresponding PC phase transition temperature, using the data compiled by Silvius (1982) as shown in Figure 40. For the straight-chain saturated family of fatty acyl chains, the relationship between increasing values of E_0 with increasing chain length and progressively increasing values of T_m is quite pronounced. This relationship establishes the validity of the first assumption, that E_0 and T_m are directly related. Within any one family of fatty acid structures such as the *iso*-branched, *anteiso*-branched or *cis*-monounsaturated, the same relationship between increasing values of E_0 with increasing chain length and increasing T_m holds. However, it is abundantly clear that when comparing across families of fatty acyl structures the same relationship between E_0 and T_m does *not* pertain. For example, altering a straight-chain to an *iso*- and then an *anteiso*-methyl branched and then to a *cis* Δ 9-monounsaturated structure drastically reduces the T_m without any comparable decrease in E_0 . Similarly, altering the position of a *cis*-double bond along the length of an 18 carbon chain has a profound effect upon T_m but is of only marginal consequence to the value of E_0 . This failure of the model to account for the relationship between specific structural substituents and T_m must lie within the second or third assumption.

Assuming for the moment that the interchain spacing, d , remains unaffected by the presence of fatty acyl structural substituents, it can then be inferred that E_0 total is not

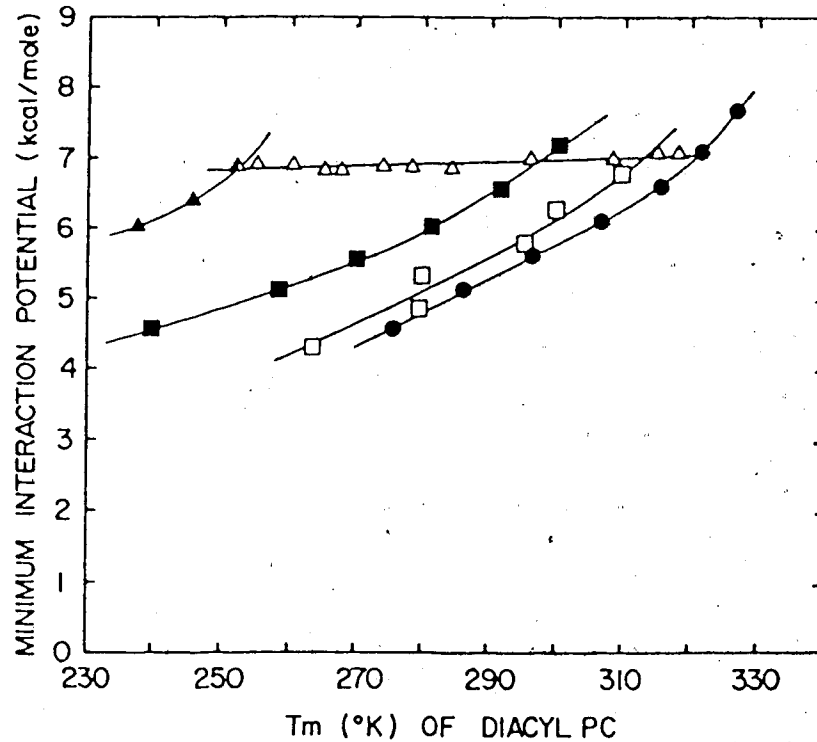


Figure 40: Minimum interaction energy for various families of fatty acid structures versus the temperature of the lipid phase transition of the corresponding PC. Values of E_0 were calculated using the data of Shapiro and Ohki (1974). T_m (PC) data were compiled by Silvius (1982). Straight-chain saturates-closed circles, methyl iso-branched-open squares, anteiso-branched-closed squares, cis- $\Delta 9$ -monounsaturates-closed triangles, isomeric cis-octadecenoates-open triangles.

equal to but is less than the sum of the interaction energies of the two constituent all-*trans* segments. That is to say that, the structural substituent perturbs the local chain packing in such a fashion as to reduce the interaction energy of methylene segments above and below its position along the chain. In order to correct the E_0 values, it would be necessary that a methyl *iso*-branch perturb up to 2 methylene segments, a methyl *anteiso*-branch perturb up to 4 methylene segments and a *cis* Δ^9 -double bond perturb up to 9 methylene segments in total.

If instead it is assumed that the presence of structural substituents in an acyl chain alters only the interchain distance, d , then it must be inferred that the quantity d varies in the order straight-chain < *iso*-branched < *anteiso*-branched < *cis*-monounsaturated, since E_0 decreases with increasing interchain distance. Moreover, the position of the structural substituent must also influence the value of d in order to account for the 18:1c Δ^x data, having its greatest effect when located near the center of the acyl chain.

The ^{19}F -NMR orientational order studies described herein support the contention that specific structural substituents have both local and overall effects on fatty acyl chain-packing, particularly in the gel-state. The local perturbing effect of structural substituents can be observed in the gel-state ^{19}F -NMR order profiles of *A. laidlawii* B membranes containing a variety of fatty acid structures.

Similarly, the relative overall ordering in the gel-state observed via ^{19}F -NMR (*cis*-monounsaturated < *anteiso*-branched < *iso*-branched < straight-chain) is exactly that expected if the relative interchain distances inferred from the above model are correct. It seems most reasonable that the shortcomings in this simple model of the relationship between chain interactions and T_m reside in the failure to account for both local and general overall effects of specific structural substituents upon chain-chain interactions. When these considerations are taken into account, the relationship between chain interaction energies and T_m is most satisfactory.

When examined from the perspective of biological relevance, the generalized picture of the relationship between fatty acid structure, orientational order and lipid phase state which has been presented in this last section raises several points of further significance. At 310°K , a plot of S_{m01} versus T_m or $(T - T_m)$ indicates that those fatty acids which most decrease the temperature of the phase transition display the lowest overall orientational order. Although in absolute terms the differences in orientational order are small, in percentage terms there exists an approximately 25% difference in ordering between the most and the least ordered situation. Thus a consequence of lowering the lipid phase transition temperature is to disorder the liquid-crystalline state at physiological temperatures. It has been estimated that, at some point, an

excessively disordered membrane will begin to display negative physiological effects.

It does not appear to be a straightforward proposition to define a unique degree of ordering below which the membrane lipids may be said to be "hyperfluid". With increasing temperature, in any one case of enrichment with a particular fatty acid, orientational order decreases in a more or less linear fashion. There is then no temperature above which order abruptly decreases. Defining a "hyperfluid" point will therefore require coordinated physiological and physical experiments. One such experiment might entail measurements of growth rates and yields at different temperatures combined with an evaluation of membrane lipid ordering under conditions of enrichment with potentially hyperfluidizing fatty acids.

Concluding Remarks

There are few research studies which provide unequivocal answers to all the questions envisaged upon their inception, while it is to be hoped that those answers which were obtained should raise new and significant questions requiring further research. In concluding, I would like to address what I consider to be the area of most uncertainty regarding the outcome of this research, as well as to indicate promising paths of investigation which follow from the results obtained.

The two areas of ambiguity which could most affect the conclusions drawn from these studies are the estimated value of the maximum chemical shift anisotropy for a MFPA in an all-*trans* configuration, and the assumption of effective axial symmetry in the gel-state. Although these two points were treated on the basis of sound theoretical considerations and indirect experimental observations, there is no replacement for direct experimentation. In fact, a single experiment should be able to resolve any doubts concerning both questions. A study of the orientation dependence of the chemical shift in macroscopically oriented multi-layers of MFPA-containing lipids should provide the answers. If the position of the fluorine resonance line was still orientation dependent at temperatures below the lipid phase transition (i.e., a $\langle 3\cos^2\theta - 1 \rangle$ dependency), then the assumption of effective axial symmetry in the gel-state would no longer be an assumption. Furthermore, the value of $(\sigma_{||} - \sigma_{\perp})_{\max}$ could be measured directly from the position of the fluorine resonance line at orientations of 0° and 90° of the oriented multilayers with respect to the magnetic field. Resolution of these questions would immeasurably strengthen the conclusions obtained in this research.

Turning to those questions raised by the outcome of these studies, it can be immediately stated that of all the literally hundreds of possible fatty acyl structural variants, only a few representative structures have been investigated. The controversial question of the effects of

fatty acyl chain length on volume versus area in a lipid bilayer may well be amenable to study via ^{19}F -NMR. The conformation and dynamics of many naturally occurring fatty acid structures such as polyunsaturated and polymethyl-branched residues remains at best uncertain. Furthermore, many of the naturally occurring fatty acyl species such as methyl-branched chain fatty acids exhibit multiple endothermic transitions when incorporated into PC's, in addition to a gel to liquid-crystalline phase transition. It would be of particular interest to ascertain whether the disordered gel-state exhibited via ^{19}F -NMR in membranes of *A. laidlawii* B enriched with methyl-branched fatty acids corresponds to a particular intermediate gel-state in the homologous PC.

Of more immediate biological concern are the physical consequences of "hyperfluidity", referring to a temperature at which a lipid bilayer becomes incapable of correct functioning. At what degree of acyl chain disordering does this situation prevail? ^{19}F -NMR results suggest that at higher temperatures ordering is a function of temperature alone, being little influenced by either fatty acid or head-group structure, at least for *A. laidlawii* B membranes. Does a lipid bilayer membrane become "hyperfluid" because the temperature has exceeded the ability of an organism to influence bilayer stability structurally? How does the "hyperfluid point" vary from lipid structure to lipid structure?

A further potential for ^{19}F -NMR studies of lipid membranes lies in the as yet largely unexploited ability to simultaneously and independently monitor the physical state of both-membrane lipids and proteins when the two are fluorine labelled. The entire field of lipid-protein interactions is burgeoning and such a capability should prove useful.

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