REVIEW LETTER

DYNAMICS OF LIPIDS IN MEMBRANES: HETEROGENEITY AND THE ROLE OF CHOLESTEROL

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Introduction

The purpose of this Review Letter is to summarise some recent advances in our understanding of the molecular dynamics of lipids in both natural and model membrane systems, which have been obtained using the techniques of differential scanning calorimetry, nuclear magnetic resonance, electron spin resonance, X-ray diffraction, infra-red and Raman spectroscopy. References to the basic principles of the techniques themselves are given at the end of this letter*.

We treat lipid–lipid and lipid–cholesterol interactions in model systems initially, and follow this by a discussion of lipid mobility in biomembranes themselves.

There is now increasing evidence that some membranes contain heterogeneous (gel and liquid crystalline) lipid domains, and the evidence for and possible implications of this are discussed.

1. Model Membranes

1.1. *Thermal Studies*

The lipids present in biological membranes, e.g. phospholipids and glycolipids, generally exhibit both thermotropic and lyotropic mesomorphism, and often form bilayer leaflet membrane structures in H_2O. Order → disorder (crystal → liquid crystal) phase transitions have been studied using both the techniques of differential thermal analysis and differential scanning calorimetry. The temperatures at which the phase transitions occur are dependent upon the headgroup, the hydrocarbon chain length, and the degree and type of unsaturation present [1]. For the same headgroup and extent of hydration, lipids with more unsaturated chains have lower transition temperatures than more saturated ones [2], longer chains higher transition temperatures than shorter ones [3], and *cis*-unsaturated chains lower transition temperatures than *trans*-unsaturated ones [4].

The mixing properties of various homologous lipids have been studied, though only in a preliminary manner [5]. Since biomembranes contain a varied population of chain lengths with different degrees of unsaturation and substitution together with (in general) a wide variety of polar headgroups, it is important to understand the phase behaviour of these mixed systems.

With widely dissimilar chain lengths, phase behaviour characteristic of a monotectic system [5, 6] is found, e.g. mixing of equimolar quantities of dioleyl lecithin with dibehenoyl lecithin results in transitions occurring at $T_c = -22\ ^\circ C$ and $T_{max} = 69\ ^\circ C$. With closer chain lengths, e.g. nC_{14} and nC_{18}, a mixed solid phase and solid plus liquid crystalline phase is present in the phase diagram. With nC_{16} and nC_{18} or nC_{14} and nC_{16}, ideal mixing in both phases occurs [5, 7]

Mixing of different polar headgroup containing lipids, e.g. cerebroside (a sugar lipid, $T_{max} = 65\ ^\circ C$) with equimolar egg lecithin ($T_{max} \simeq -5\ ^\circ C$), results in a mixed gel–liquid crystal system with a lower ($\simeq 35\ ^\circ C$)T_{max} [8]. The mean 'fluidity' of the system is thus higher than that of the pure cerebroside but lower than that of the egg lecithin, at the same

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* See appendix for abbreviations.

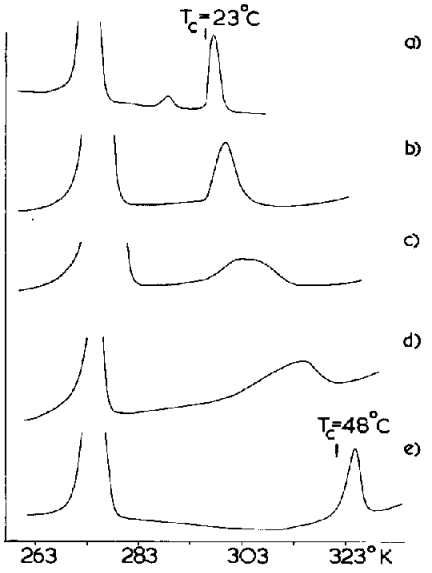


Fig. 1. Differential scanning calorimetry thermograms of dimyristoyl lecithin (DML)–dimyristoyl phosphatidyl ethanolamine (DMPE) mixtures. a) DML 100 mole %; b) DML 90 mole % + DMPE 10 mole %; c) DML 70 mole % + DMPE 30 mole%; d) DML 50 mole % + DMPE 50 mole %; e) DMPE 100 mole %; all in excess water (Keough and Chapman [9]).

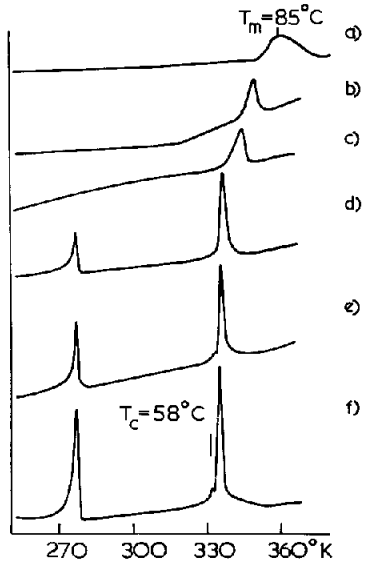


Fig. 2. Differential scanning calorimetry thermograms of distearoyl lecithin as a function of hydration: a) anhydrous; b) 10 wt % H_2O; c) 20 wt % H_2O; d) 25 wt % H_2O; e) 30 wt % H_2O; f) 40 wt % H_2O (Williams and Chapman [1]).

temperature. Similar behaviour is found in the system dimyristoyl lecithin–dimyristoyl phosphatidyl ethanolamine, fig. 1. Dimyristoyl lecithin has T_c = 23 °C (fig. 1a) and for dimyristoyl phosphatidyl ethanolamine T_c = 48 °C (fig. 1e). The pretransitional peak of the lecithin, thought to be due to a rearrangement of the polar headgroup, is removed on addition of small amounts of the dimyristoyl phosphatidyl ethanolamine (fig. 1b) [9]. At higher concentrations (fig. 1c, d), the endotherm is very broad. This indicates the presence of clusters of gel and liquid crystalline lipid in the bilayer, and the reduction in enthalpy of the transition is characteristic of a lower cooperativity of the transition in the mixture compared to the individual components. Such broad transitions are typical of several biomembranes (*vide infra*).

The effect of water has also been shown to have a profound effect on the temperature and enthalpy of the gel → liquid crystal phase transition [3]. DSC curves of distearoyl phosphatidylcholine indicate that 10 moles of water per mole of lecithin are bound, i.e. are unfreezable, at 0 °C [10]. Addition of water to the anhydrous material results in hydration of the polar headgroups and this causes a lowering, fig. 2, of the transition temperature. It is also apparent that the *shape* of the phase transition endotherm alters. The transition changes to a *highly* cooperative phenomenon on hydration, consistent with laser-Raman evidence [11], and the increased enthalpy of the transition [3].

The effect of cholesterol on the gel → liquid crystal transition of several lipids has been studied. The addition of cholesterol to dipalmitoyl lecithin in water (fig. 3) causes a lowering of the transition temperature over and above 20 mole % cholesterol and a decrease in the heat of transition [2]. At high (50 mole %) cholesterol concentrations the DSC endotherm is completely removed. A condition of 'intermediate fluidity' is produced. Below T_c, in the presence of cholesterol, the chains are more mobile than in the absence of cholesterol, and above T_c, they are less mobile. Above T_c the steroid nucleus effectively prevents flexing of the lipid hydrocarbon chains, and below T_c, it prevents them from crystallising into the rigid α-crystalline gel condition. Although the transition from gel → liquid crystal is not detectable by DSC, laser Raman evidence indicates that a transi-

tion *still* takes place, though over a very wide temperature range, and it is now a *non-cooperative* phenomenon [11]. It has also been suggested recently that at low cholesterol concentrations, lecithin–cholesterol complex-formation can occur [12].

The removal of gel → liquid crystal transitions by cholesterol is not restricted to lecithin, and has been demonstrated for other phospholipids (e.g. sphingomyelin [13]) and glycolipids (e.g. cerebroside [13]). The effect of cholesterol on the 'bound water' has also been discussed [2].

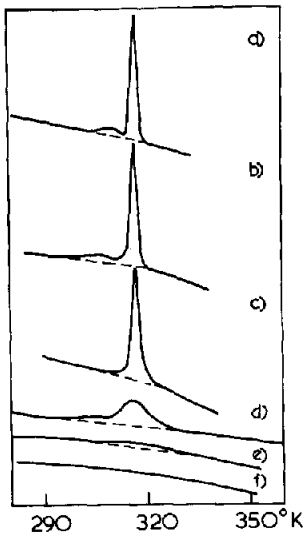


Fig. 3. Differential scanning calorimetry thermograms of 50 wt % dispersions in water of dipalmitoyl lecithin–cholesterol mixtures containing: a) 0 mole %; b) 5 mole %; c) 12.5 mole %; d) 20 mole %; e) 32 mole %; f) 50 mole % cholesterol (Ladbrooke et al. [2]).

1.2. *Nuclear Magnetic Resonance*

1.2.1. Proton Wide-Line and Pulsed NMR

Early wide-line studies indicated that NMR was a promising technique with which to study molecular mobility in membrane systems [14]. Anhydrous lecithins at liquid nitrogen temperatures were shown to have chain proton linewidths of ~ 1.6 mT* and this was gradually reduced on heating to 0.8 mT (distearoyl lecithin), [15] or in the presence of water, 0.4 mT [16], in the α-crystalline gel phase. It was apparent, however, that the headgroups had considerable mobility, especially near the thermal phase transition [16].

* 1T = 1 Tesla ≡ 10 Kgauss.

In the liquid crystalline phase, linewidths of ~ 10 μT were obtained from the chain protons and choline $-CH_2CH_2-$headgroup, and lines of 0.1 mT from the more rigid glycerol backbone. In the liquid crystalline phase, it was found that the $-\overset{+}{N}Me_3$ headgroup in lecithins was highly mobile [16, 17].

As well as these frequency domain studies, time domain studies have been performed on the gel and liquid crystalline phases of lecithins and membrane lipids [17]. Spin–spin relaxation data have been interpreted in terms of a purely dipolar origin of the observed linewidths, which means that these linewidths are a good indication of the molecular mobility present in the system [17, 18], at least in fields up to ~ 2T.

Spin-lattice relaxation in the laboratory frame (T_1) [19] and rotating frame ($T_1\rho$) [20] have also been applied to studies of lipid mobility, and moderately good correlations with continuous wave data have been obtained. This aspect of spin-dynamics is, however, complicated by the possibility of spin-diffusion, which may occur in these *non-sonicated* systems. Spin-diffusion is predicted not to occur in systems giving rise to well resolved high-resolution spectra, since it essentially depends on spectral overlap of resonance lines [21] and also on the condition $T_2 \ll T_1$. That spin-diffusion does not in fact occur in sonicated liquid–crystalline lecithin has recently been experimentally confirmed by two groups [22–24]. T_1 measurements are thus more easily interpreted in terms of molecular mobility in these systems.

Some preliminary wide-line studies of lecithin–cholesterol interactions have also been reported recently [12].

1.2.2. Proton High-Resolution NMR

High-resolution NMR studies of molecular mobility were initially hampered by the low signal-to-noise obtained on ^1H at 60 MHz [25]. Increased use of higher fields (220 MHz) have effectively solved this problem, and it has been demonstrated that well resolved high-resolution proton NMR spectra from unsonicated smectic liquid crystalline lipids, can be obtained. Moderate resolution of an egg yolk lecithin spectrum has been obtained [18], though some lipids give considerably better resolved spectra. For example, sphingomyelin (T_{max} = 40 °C) at 60 °C shows, fig. 4a, $-\overset{+}{N}Me_3$, $(CH_2)_n$ and CH_3 signals [13]. In the presence

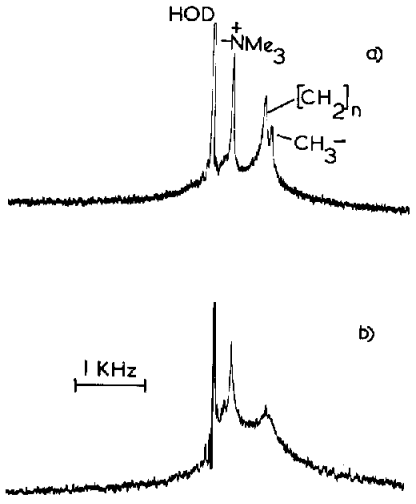


Fig. 4. 220 MHz ^{1}H-NMR spectra of 20 wt % hand-dispersions in D_2O of: a) Sphingomyelin, 60 °C; b) Sphingomyelin–cholesterol (1:1), 60 °C (Oldfield and Chapman [13]).

of equimolar quantities of cholesterol, the signals from the hydrocarbon chains are broadened, fig. 4b, which is consistent with the 'rigidising' role of cholesterol above the transition temperature range. Below this range, however, the chains are 'fluidised'. (No signal from the hydrocarbon chains is seen below ca. 30 °C for sphingomyelin). The "intermediate fluid" sphingomyelin–cholesterol state is relatively temperature insensitive over the temperature range 20 °C → 60 °C, as has been found previously for the dipalmitoyl lecithin–cholesterol system [12]. Similar results have been obtained for the system cerebroside–cholesterol (1:1) [13].

1.2.3. Carbon-13 NMR

Because of the relatively broad and overlapping signals obtained using ^{1}H-NMR, there is at present much interest in the use of ^{13}C-NMR (carbon magnetic resonance, CMR). ^{13}C has a smaller gyromagnetic ratio than ^{1}H, and is thus less susceptible to dipolar broadening. In addition, the chemical shift range is large (~ 200 ppm typically), and spin diffusion in natural abundance does not occur so that the interpretation of T_1 measurements is simplified.

The first reported [26] CMR of smectic liquid crystalline lecithin in H_2O indicated the choline-$\dot{N}Me_3$ group was relatively mobile, though unfortunately a broad envelope of resonances for the methylene carbons was still obtained. However, unsaturated CH=CH residues in the hydrocarbon chains were well resolved from the main envelope. It would thus appear that T_1 measurements on these defined resonances should give more information about molecular mobility in these systems. The effect of cholesterol, using CMR, has not yet been reported.

1.2.4. Deuteron Magnetic Resonance

Nuclei with spin $I \geqslant I$ have an associated electric quadrupole moment. Under some circumstances, this gives rise to a well defined splitting of the nuclear Zeeman levels. For deuterium, I = 1 and the observed splitting of the NMR absorption line is [27]

$$\Delta\nu_{max} = \frac{3}{4}\frac{e^2qQ}{h}\langle 3\cos^2\theta - 1\rangle$$

where e^2qQ is the quadrupole coupling constant (170 KHz for CD_2 groups [28]) and θ is the angle between the laboratory field and the electric field gradient tensor at the nucleus.

Chain deuterated dimyristoyl lecithin

$$\begin{array}{l} CH_2OCO(CD_2)_{12}CD_3 \\ | \\ CHOCO(CD_2)_{12}CD_3 \\ | \\ CH_2OP\bar{O}_2OCH_2CH_2\dot{N}Me_3 \end{array}$$

(in its gel state) at 10 °C gives a broad spectrum, fig. 5a, composed of overlapping CD_2 group doublets [29], implying that different groups are subject to different rates or types of motion, down the chain. Just above the transition at 23.5 °C, a maximal quadrupole splitting $\Delta\nu_{max}$ = 29.8 ± 1 KHz is apparent, and at 30 °C the splitting is 27 ± 1 KHz (fig. 5b, c). From the shape of the spectrum it is apparent that a relatively wide correlation time distribution is present along the alkyl chains. CD_2 groups near the polar/apolar interface are relatively restricted in their molecular motion, whilst those at the methyl terminal end have greater mobility. This is consistent with high resolution NMR evidence [18].

Addition of cholesterol to the dimyristoyl lecithin at 30 °C, in molar ratios, causes an increase in the maximal quadrupole splitting from 27 ± 1 KHz to, fig. 5d, 49.4 ± 1.5 KHz. This means that $\langle 3\cos^2\theta - 1\rangle$

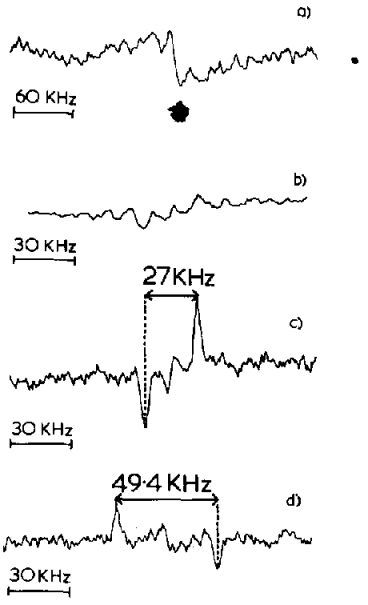


Fig. 5. 8 MHz Deuteron magnetic resonance spectra of di(perdeutero) myristoyl lecithin–H_2O at: a) 10 °C; b) 23.5 °C; c) 30 °C; d) 30 °C, lecithin-cholesterol (1:1) (Oldfield et al. [29].

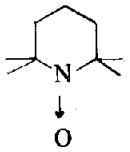
is quite large, i.e. that the motion of a larger number of the acyl chain deuterons is highly restricted. A central narrower component is still present however [29, 12] implying that the groups near the Me-end of the chain are not appreciably affected in their motion by the presence of the bulky steroid nucleus. This interpretation agrees well with data using other techniques [30].

Cooling the lecithin–cholesterol system below T_c for the lipid alone, to 10 °C, results in an increase in the maximal quadrupole splitting to 53.2 ± 1.5 KHz – a slight decrease in mobility. This is consistent with the intermediate fluid hypothesis with a relatively temperature-insensitive lecithin–cholesterol "complex" present.

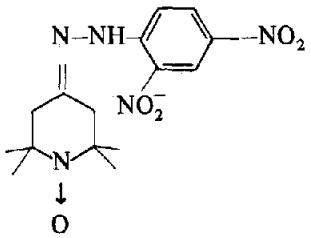
1.3. *Electron Spin Resonance Studies*

The technique of spin-labeling with nitroxide substituent bearing molecules introduced by McConnell and coworkers [31, 32], has found widespread application in model-membrane structure studies. Three basic types of experiment have been performed – i) solubility studies; ii) studies on the correlation time distribution along spin-labelled molecules and iii) diffusion of spin-label studies.

i) The nitroxide TEMPO (2,2,6,6-tetramethyl piperidine-1-oxyl)

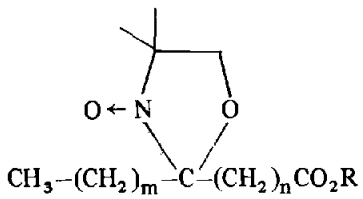


is water soluble. It has been used to study the gel → liquid crystal phase transition of dipalmitoyl lecithin in H_2O, since it has a high solubility in the fluid liquid crystal, and a low solubility in the gel [30]. The label



(the 2,4-dinitrophenylhydrazone derivative of 2,2,6,6-tetramethylpiperid-4-one-1-oxyl) has also been used in this way to monitor the gel → liquid crystal phase transition of dipalmitoyl lecithin [33]. This partitioning effect of TEMPO has recently been suggested by McFarland [34] to be promising as a probe technique for assaying the amount of "fluid" regions in biomembranes, and it has been shown that at least 65% of the lipids in sarcoplasmic reticulum membranes are in a fluid state [34].

ii) Mobility in bilayers. Spin labelled fatty acid derivatives of the general type



(R = H, Me or a lipid residue)

have been used to study the rates and types of motion at different regions along the hydrocarbon chains in

bilayers in both liposome dispersions [30, 35, 36] and in oriented systems [37].

When spin labelled fatty acids or lecithins are incorporated into liquid crystalline egg lecithin bilayers, it is found that the decrease in order parameters S (defined as = 0 for an isotropic liquid and = 1 for a perfect crystalline solid) is greater than logarithmic with increasing n, the number of methylene groups separating the label from the polar headgroup [30]. This increased motion towards the centre of the bilayer is difficult to reconcile with a model in which the hydrocarbon chains are in a parallel array, and it has been suggested, using a spin-labelled phospholipid label, that a net tilt of $\sim 30^{\circ}$ is present in the headgroup region of hydrated egg lecithin multilayers [36], producing a carbon atom density ~ 12% higher than the carbon atom density near the terminal methyl groups – which is the order of magnitude density change observed between liquid hexadecane ($\rho = 0.77$) and solid paraffin ($\rho = 0.88$). Calculations have suggested this tilted region has a lifetime of greater than 10^{-8} sec [36]. The extent of this tilted region is expected to be dependent on many structural features of the lipid, and the temperature.

In the presence of cholesterol (egg lecithin:cholesterol, 2:1) it has been shown that the *first 8 carbon atoms* from the bilayer surface can be thought of as a *rigid rod* [30], with the remaining carbons greatly increasing their motion towards the centre of the bilayer. It has also been demonstrated that cholesterol can have a 'dual' role in formation of an intermediate-fluid state with several types of lipid class [35].

Using the label methyl 4-(2′-(*N*-oxyl-4′,4′-dimethyl oxazolidine))–stearate (4NS), the unpaired electron in which residues predominantly at the polar/apolar interface, values of twice the maximal hyperfine splitting ($2T_m$) of 6.1 mT in the gel state of dipalmitoyl lecithin at 20 °C, and 4.1 mT in the fluid liquid crystalline state of egg lecithin, fig. 6a, b, have been reported [35]. Addition of equimolar quantities of cholesterol results in very similar spectral lineshapes, fig. 6c, d, with the dipalmitoyl lecithin–cholesterol system having $2T_m$ = 5.65 mT and the egg yolk lecithin–cholesterol system $2T_m$ = 5.27 mT. This is consistent with an intermediate fluid liquid crystalline state being formed from the rigid gel of dipalmitoyl lecithin and the fluid liquid crystal of egg yolk lecithin. Noticeably, the egg yolk lecithin–cholesterol

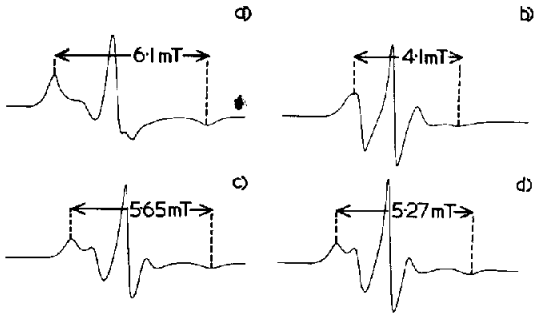


Fig. 6. Electron spin resonance spectra (X-band) of the spin-label methyl 4-(2′-(*N*-oxyl-4′,4′-dimethyl oxazolidine)) – stearate in 7 wt % aqueous hand-dispersions of: a) dipalmitoyl lecithin, 20 °C; b) egg yolk lecithin, 20 °C; c) dipalmitoyl lecithin–cholesterol (1:1), 20 °C; d) egg yolk lecithin–cholesterol (1:1), 20 °C (Oldfield and Chapman [35]).

system is *more fluid* than the dipalmitoyl lecithin–cholesterol system at the same temperature (5,27 mT egg yolk lecithin:cholesterol, 5.65 mT dipalmitoyl lecithin–cholesterol). These interesting results are borne out when the label 12 NS is used. In dipalmitoyl lecithin at 20 °C, $2T_m$ = 5.7 mT, and in egg yolk lecithin $2a_N$ (twice the isotropic hyperfine splitting) = 2.8 mT. Addition of cholesterol causes fluidisation of the dipalmitoyl lecithin ($2T_m$ = 4.85 mT), and immobilisation of the egg yolk lecithin ($2T_m$ = 4.15 mT). The egg yolk lecithin:cholesterol system is more fluid than the dipalmitoyl lecithin:cholesterol system, at the same temperature.

Near the polar groups the mobility or order of the chains in gel and intermediate fluid liquid crystal is similar (dipalmitoyl lecithin/H_2O/4NS,$2T_m$ = 6.1 mT, dipalmitoyl lecithin:cholesterol (1:1)/H_2O/4NS $2T_m$ = 5.65 mT, ΔT = 0.45 mT) whereas near the methyl group end of the chains the mobility is quite dissimilar in gel and intermediate fluid liquid crystal, (dipalmitoyl lecithin/H_2O/12NS, $2T_m$ = 5.7 mT, dipalmitoyl lecithin:cholesterol (1:1)/H_2O/12NS, $2T_m$ = 4.85 mT, ΔT = 0.85 mT). The interpretation of rigid hydrocarbon chains near the polar/apolar interface in the presence of cholesterol is consistent with the available wide-line NMR data [12, 29]. Quantitative comparison between ESR and NMR data is difficult, and two recent NMR reports [38, 39] have suggested that ESR results may somewhat overestimate the width of

the correlation time distribution in liquid crystalline lecithin, so that ESR results should perhaps be viewed as essentially a qualitative reflection of the mobility of the unlabelled molecule.

iii) Diffusion experiments

a) Flip-flop across a bilayer

The exchange of a spin-labelled phospholipid across a lipid bilayer (single bilayered vesicles produced by prolonged sonic irradiation) has been measured by selectively destroying label on one side of the vesicle by ascorbate [40]. The exchange rate was measured and was found to be slower than 2×10^{-5} times/sec.

b) Lateral diffusion

Using a headgroup spin-labelled lecithin in sonicated single bilayer vesicles of di(dihydrosterculoyl)-lecithin (sonication in the presence of unsaturated lipids causes rapid loss of label paramagnetism), it has been shown that by analysis of the line-broadening of the $-\overset{+}{N}Me_3$ group caused by the presence of the label, that the frequency of molecular jumps leading to lateral diffusion must be greater than 3×10^3 jumps/sec [41].

1.4. *X-Ray*

Single crystal structure determinations on synthetic phospholipids have not yet been reported, most work having been powder studies.

The mesomorphic properties of diacylphosphatidyl-ethanolamines, phosphatidylcholines and phosphatidyl-serines have all been thoroughly studied [10]. On transformation of an anhydrous crystalline material into a liquid crystalline mesophase, long spacings decrease (e.g. with dimyristoyl phosphatidylethanol-amine the spacing decreases from 49.9 Å to 34.5 Å) [4]. This is accompanied by a change in short spacing, giving a diffuse 4.6 Å line characteristic of liquid hydro-carbons. These two observations are consistent with a *relatively* disordered hydrocarbon interior.

Powder studies on anhydrous natural phospholipids, and on numerous phospholipid water systems (see for example [1, 10]) have been reported.

1,2-Dipalmitoyl-L-lecithin:cholesterol:water systems show only integral orders of a principal long spacing in the low-angle region. At constant lipid/water ratio at 25 °C, there is a large increase in this long spacing on addition of cholesterol, which reaches a maximum of 81 Å at 7.5 mole % cholesterol. There is no observable change in the short spacing which remains at 4.2 Å (characteristic of crystalline paraffinic chains). On further addition of cholesterol, there is a gradual decrease in long spacing to 64 Å which is accompanied by a change in the short spacing to 4.45 Å, and this spacing becomes diffuse [2].

These results correspond to

a) a straightening of the hexagonally packed gel hydrocarbon chains, up to 7.5 mole % cholesterol, combined with an increase in the thickness of the water layer of 9 Å, to 27.5 Å and

b) a fluidisation of the hydrocarbon chains between 7.5 and 50 mole % cholesterol, as indicated by the 4.2 Å sharp high angle spacing changing to a 4.45 Å (diffuse) spacing, together with a decrease in the long spacing of 17 Å due to the increased lateral motions of the chains. These latter ideas are consistent with the available ESR, NMR and calorimetric evidence [35, 12, 2]. Between 7.5 and 42 mole % cholesterol the water layer thickness is constant since 30 wt % water can be taken up by both systems. The amount of unfreezable "bound" water has been shown calori-metrically to be constant from 0–7.5 mole % cholester-ol, and then to increase (with constant water layer thickness) between 7.5 and 50 mole % cholesterol.

1.5. *Infra-Red and Laser-Raman Studies*

1.5.1. Infra-Red Spectroscopy

Early work indicated that infra-red spectroscopy was an excellent technique for studying hydrocarbon chain mobility in lipids, and for the study of their thermotropic mesomorphism. The importance of cis/trans isomerism in determining transition tempera-tures was demonstrated with the dielaidoyl (*trans*-octadec-9-ene-oyl) and dioleyl (*cis*-octadec-9-ene-oyl) phosphatidylethanolamines [4]. The former is crystalline at room temperature and the latter is liquid crystalline, and this results in broadened absorption bands characteristic of liquid films. The band at 720 cm^{-1}, assigned to a rock mode of 4 or more all trans CH_2 groups, decreases in intensity on going from crystal → liquid crystal, and continues to decrease in intensity on further heating, due to the larger number of conformers occurring at higher tempera-tures.

Infra-red studies in water or D_2O are difficult, due to overlapping solvent bands, however, preliminary results have been reported with phosphatidylethanol-amines at low water concentrations [42].

ii) Raman Spectroscopy

A particular advantage of (laser) Raman spectroscopy is that the effect depends on the change in polarisability of the molecule during a vibration. Symmetric vibrations that are infra-red inactive can thus be seen in the Raman spectrum. In addition the absorption due to H_2O around 1600 cm^{-1} is of low intensity.

Laser Raman studies of the thermal phase transitions in lecithin and lecithin–cholesterol [11] have shed light on the cooperativity of the transition. Anhydrous dipalmitoyl lecithin has been suggested to undergo a non-cooperative thermal phase transition (a *broad* thermal phase transition is observed for anhydrous distearoyl lecithin, fig. 2a), as does dipalmitoyl lecithin–cholesterol (1:1), in excess water. The low cooperatively of the gel → liquid crystal phase transition at intermediate cholesterol concentrations has also been suggested recently by Trauble [43].

Hopefully, by use of deuterium substitution, it should be possible to shift *selected* resonances to much lower energies so that it might be possible to detect the motions of different parts of a molecule in similar, or even more complex systems.

2. Some Recent Studies on Biomembranes With High Cholesterol Levels

2.1. *Thermal Studies*

Differential scanning calorimetry studies of both myelin and erythrocyte membrane lipids have been reported [2, 44]. With myelin [2], it was shown that no thermal transition was obtained from 'intact' membranes in H_2O, or from total lipids in excess water. Removal of cholesterol from the total lipids resulted in a *broad* DSC transition encompassing the physiological 37 °C, to be observed. This indicates that gel and liquid crystalline regions are present in the cholesterol depleted lipids, and that an effect of cholesterol is to remove or fluidize the gel lipid areas (and presumably to make the liquid crystalline regions present, less fluid). Dehydration of the membranes likewise caused a DSC endotherm to be observed, since crystallisation of the cholesterol occurred, indicating the importance of water in preserving membrane structure.

Erythrocyte ghosts do not show a phase transition by DSC, although cholesterol depleted lipids do. The observed transition encompasses 37 °C. However, an additional small endotherm at low temperatures has been detected [10] and attributed to the presence of highly unsaturated species i.e. the lipids form a kind of monotectic system [5, 6]. In the presence of cholesterol, no endotherms are detected from the lipids. Cholesterol may thus have a *dual role* of preventing formation of crystalline gel areas in some membranes whilst also inhibiting the motion of hydrocarbon chains in more fluid, liquid crystalline, regions.

2.2. *Nuclear Magnetic Resonance Studies*

2.2.1. Proton Wide-Line NMR

Wide-line studies of myelin and erythrocytes have been reported [16, 44] and show the presence of very wide lines of ca. 0.2 mT and 0.5 mT (myelin) and 0.2, 0.3, 0.34 and 0.58 mT (erythrocytes), similar to those seen in the lecithin–cholesterol system [12], and they may have similar origins.

2.2.2. Proton High-Resolution NMR

It has been reported [45] using 220 MHz proton NMR that the choline–$\overset{+}{N}Me_3$ group in myelin is resolvable, and hence mobile, similar results have been reported for erythrocyte ghosts at 31 °C [46]. At 18 °C [47] no spectrum is observed, though at 75° reversible dissociation of polypeptides from the membrane occurs [48]. Care must thus be taken in the treatment of membranes, since it is also known that many ghost proteins are water soluble [49].

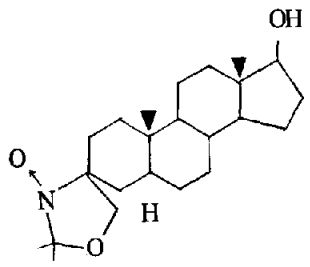
Studies on ultrasonically dispersed membranes have also been reported [44, 50] and well resolved spectra have been obtained.

2.2.3. Carbon-13 NMR

Carbon-13 studies of erythrocyte membranes have been reported [51]. The resolution is better than that obtained from proton NMR, and again appears to indicate relatively mobile choline–$\overset{+}{N}Me_3$ groups.

2.3. *Electron Spin Resonance*

Spin labeling using labels of the fatty acid or steroid type e.g. the isoxazolidine derivative of androstan-3-one-17-β-ol



have been used to investigate mobility in erythrocyte ghosts [52] as has the label TEMPO [53]. TEMPO has a low solubility in erythrocyte membranes, consistent with a large proportion of relatively highly ordered lipid domains. In shear-oriented erythrocyte ghosts [52] the *N*-oxyl-4′,4′-dimethyl-oxazolidine derivatives of 5α-androstan-3-one-17β-ol and sodium 12-ketostearate showed moderately high spectral anisotropy. Both spectra were more immobilised than in model sonicated phosphatide dispersions, again indicating possible lipid–cholesterol and/or lipid–protein interactions.

The effect of cholesterol on the mobility of cholestane spin-labeled cholesterol-depleted brain lipids has been reported recently [54]. It was suggested that the cholesterol caused the hydrocarbon chains to become more 'ordered'. It may be that there is some preference of the spin label to probe the more fluid liquid–crystalline regions in this type of system [13, 55], i.e. spin-label in *liquid-crystalline* regions becomes more ordered.

2.4. *X-Ray*

Recent studies have indicated that erythrocyte membranes contain a bilayer structure, as does myelin [56].

2.5. *Infra-Red Studies*

Infra-red studies of myelin and of erythrocyte ghosts, and of their extracted lipids have been reported [44, 57, 58].

Erythrocyte lipids show a prominent 720 cm^{-1} band due to a $(CH_2)_{n\geq 4}$ rock mode, consistent with relatively ordered segments of hydrocarbon chain; this is in the presence of cholesterol. Intact membranes lack this distinctive feature, indicating possible lipid–protein interaction.

3. Some Recent Studies on Biomembranes With Low Cholesterol Levels

3.1. *Thermal Studies*

It has been shown that the 'intact' plasma membranes and isolated lipids of *Acholeplasma laidlawii* B exhibit thermal phase transitions from gel → liquid crystal [59–61]. These transitions are all broad, extending over ~ 30 °C, and thus are of low cooperativity. The thermal transitions of the isolated lipids, fig. 7, closely resemble the transitions of the membranes themselves [59]. Grown in unsupplemented media, the transition range encompasses the growth temperature [59, 60]. This means that *at the growth temperature, both rigid crystalline gel and fluid liquid crystalline regions are present in the membrane lipids.* A similar phenomenon has been observed with the membranes of *Escherichia coli* [62], where for organisms grown at 37 °C the thermal transition extends from ~ 15 °C → 45 °C. Again, gel and liquid crystalline regions are present in the membrane lipids *at their growth temperature.* This interpretation is supported by recent X-ray results [63].

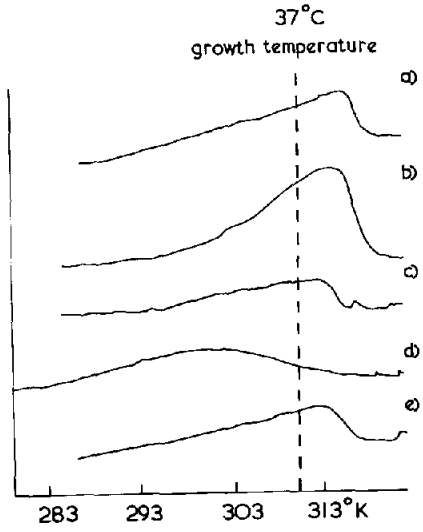


Fig. 7. Differential scanning calorimetry thermograms of 50 wt % dispersions of lipids extracted from *A. laidlawii* B: a) total lipids; b) glycolipids; c) phospholipids; d) neutral lipids; e) reconstituted total lipids (Chapman and Urbina [61]).

3.2. *Nuclear Magnetic Resonance Studies*

Support for the existence of predominantly *rigid* hydrocarbon chains in the membranes of *Acholeplasma laidlawii* B membranes comes from deuteron NMR [64]. Cells supplemented with deuterated palmitic acid or lauric acid (which is elongated to myristic and palmitic acids) show DMR spectra, fig. 8, more characteristic of the gel state of the model system di(perdeutero)myristoyl lecithin, than the liquid crystalline state of this lipid, or of the model smectic liquid crystalline soap, potassium perdeuterolaurate–H_2O. ESR spin-labeling results, however, give spectra characteristic of a mobile nitroxide – indicating some preference of the label for the more fluid, liquid crystalline regions of this heterogenous membrane system [65].

That rapid growth can occur with almost all the membrane lipids in a rigid gel state is suprising, though the results of Steim et al. [59] indicate that with stearate supplemented *A. laidlawii* B, that is the case.

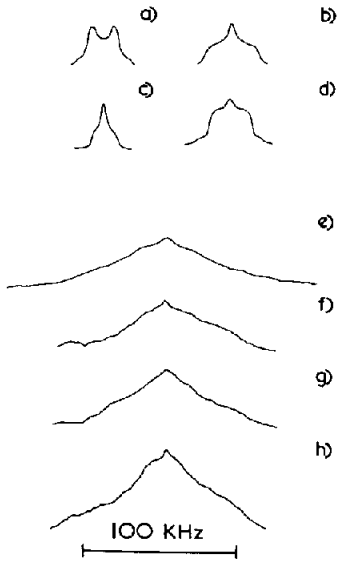


Fig. 8. Theoretical and experimental deuteron magnetic resonance spectral lineshapes: a) theoretical powder lineshape, $\eta = 0$; b) $\eta = 1.0$; c) absorption spectrum of potassium perdeuterolaurate 70 wt %–H_2O, 30 °C, (smectic liquid crystalline); d) di(perdeutero)myristoyl lecithin 5 wt %–H_2O, 30 °C, (smectic liquid crystalline); e) 10 °C (gel state) f) *A. laidlawii* B membranes, supplemented with perdeuterolauric acid 30 °C, spectrum recorded at 30 °C; g) supplemented with perdeuteropalmitic acid at 30 °C, spectrum recorded at 30 °C; h) supplemented with perdeuteropalmitic acid at 37 °C, spectrum recorded at 37 °C (Oldfield et al. [64]).

3.3. *Electron spin resonance studies*

Spectra characteristic of highly mobile nitroxides have been obtained from a wide variety of membranes [66]. A central question in interpreting these results is to what extent a spin-label can detect 'rigid' regions in the presence of 'fluid' ones, i.e. what is the extent of the fluid environments, and also, can the spin-probe artifically create them? These questions are as yet not fully answered. However, indications that the 12NS spin-label may probe liquid crystalline regions in preference to gel regions have recently been obtained, in model systems [55], and that labels can act as perturbants has been inferred by other workers [38, 39, 67, 68].

3.4. *X-Ray*

X-Ray diffraction has been shown to be a powerful technique with which to obtain information on membrane heterogeneity.

Engelman [69] has shown that in palmitate supplemented *A. laidlawii* B, at the growth temperature both 4.15 Å (gel) and 4.6 Å (liquid crystalline) short spacings are obtained from the membranes. The gel region only disappears at 45 °C, i.e. *well above* the growth temperature of 37 °C.

Esfahani et al. [63] have obtained similar results on *E. coli* K12. With elaidate supplemented membranes, the X-ray detected thermal phase transition extends from 30 °C → 40 °C in intact membranes (grown at 37 °C). With linolenate and myristoleate membranes the transitions extend from 36 °C → 46 °C – here the lipids (phosphatidylethanolamines) would appear to be in predominantly a *gel* state (as evidenced by the sharp 4.2 Å band). It is interesting to note that fatty acids which might be expected to produce highly fluid lipids (i.e. linolenate, myristoleate, oleate) are taken up to a smaller extent than those expected to produce more rigid lipids e.g. elaidate [4]. There would thus appear to be a mechanism controlling fluidity which operates by increasing palmitate concentrations in the presence of linolenate etc. (Linolenate supplemented membranes contain 67% palmitate and 23% linolenate, elaidate supplemented membranes 75% elaidate and only 14% palmitate, reflecting the close physical similarities of dielaidoyl phosphatidylethanolamine and dipalmitoyl phosphatidylethanolamine [70].

All the membrane lipid phase transitions of Esfahani et al. were shown to occur at ≈37 °C. The transition temperature of dipalmitoyl phosphatidylethanolamine is ~70 °C, i.e. admixture of heterogenous lipids (and possible lipid–protein interaction) has brought the transition temperature down many degrees, and has caused formation of a *broad* transition, indicative of heterogeneity and low-cooperativity, similar to the results found with *A. laidlawii* B lipids, fig. 7, and the model lecithin-phosphatidylethanolamine system, fig. 1.

In some instances, micro-organisms lacking cholesterol may regulate 'permeability' and mechanical stability by having heterogeneous gel and liquid crystalline regions in their membranes.

The possibility that "rigid" as well as "fluid" (or "liquid-like") domains in biomembranes may be a common occurrence, would appear to be an important factor in constructing models for some biomembranes. That biologically relevant transport processes can occur in rigid systems has recently been shown by Krasne et al. [71], where it was demonstrated that the ion translocating antibiotic, gramicidin, was able to mediate potassium ion transport in both "solid" as well as "liquid" black lipid membranes.

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Appendix

1) Abbreviations

DSC : Differential scanning calorimetry.
NMR : Nuclear magnetic resonance.
DMR : Deuteron magnetic resonance.
CMR : Carbon magnetic resonance.
ESR : Electron spin resonance.
T_c : The onset temperature for a crystal → liquid crystal phase transition measured by DSC. Also, onset temperature for phase change of lower melting component in a monotectic system.
T_{max} : The maximum rate of melting of a mixed lipid system exhibiting a broad thermal phase transition; describes maximum rate of melting of second component in a monotectic system.
T : Tesla, S.I. unit equivalent to 10^4 gauss.
T_1 : Spin-lattice relaxation time: time constant describing return of spin system to thermal equilibrium after saturation. See Slichter for further discussion.
T_2 : Spin-spin relaxation time: time constant describing 'dephasing' of spins after a r.f. pulse. See Slichter for discussion.
$2T_m$: Twice the maximal hyperfine splitting observed for a spin-label, a function of the order parameter S.

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