A ^{2}H-NMR STUDY OF THE COMPARATIVE EFFECTS OF LANOSTEROL AND
CHOLESTEROL ON MODEL MEMBRANES

by

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Abstract

Suspensions of sn-2 deuterated ^{2}H-DPPC-d31 containing various concentrations of either lanosterol or cholesterol were compared using deuterium nuclear magnetic resonance spectroscopy (^{2}H-NMR) and differential scanning calorimetry (DSC). The alpha face of cholesterol is smooth whereas a hydroxyl group protrudes from the alpha face of lanosterol.

At the sterol concentrations studied, (0%, 5%, 10%,15%), the phase behaviour of lanosterol and cholesterol was similar. M_1 profiles showed that the gel to liquid crystalline phase transition is broadened with the incorporation of both sterols and that mixed phase regions exist at comparable concentrations and temperatures.

Both lanosterol and cholesterol increase the average order of the phospholipids above the transition temperature. Since both lanosterol and cholesterol produce this ordering, it is proposed that the significant structural feature producing this result is the rigidity of the cholestane ring.

T_{2e} increases with the incorporation of both cholesterol and lanosterol. This could indicate the importance of the rigid ring structure which may modify slow motions such as the collective motions of the phospholipid chains.

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I. INTRODUCTION

Cholesterol is a major component of many biological membranes. There is evidence to suggest it performs some specialized role(s) in natural membranes. Firstly, it is structurally very different from the other lipid components. Secondly, cholesterol is synthesized via a complex pathway and a significant amount of metabolic energy is expended during its formation [1]. Finally, and most significantly, cholesterol is present in equimolar concentrations with the phospholipids in most eukaryotic membranes but is in general absent from prokaryotic membranes [2].

Investigations with model membranes have shown that the presence of cholesterol influences the permeability [3-5], phase behaviour [6 and references therein] and degree of order of the lipid components in the bilayer [7,8]. These results have been interpreted in terms of two key structural features of cholesterol; the planar ring structure and the smoothness of the alpha face [9,10], (figure 1).

Lanosterol, (figure 1), is an intermediate in cholesterol biosynthesis and undergoes three sequential oxidative demethylation steps at positions 4, 4 and 14 to form cholesterol [1]. It is normally present in trace amounts [11] and does not perform any known physiological role. The 14 a methyl group which projects from the alpha face of lanosterol is thought to be the most significant structural difference between lanosterol and cholesterol and could interfere with the Van der Waal interactions between
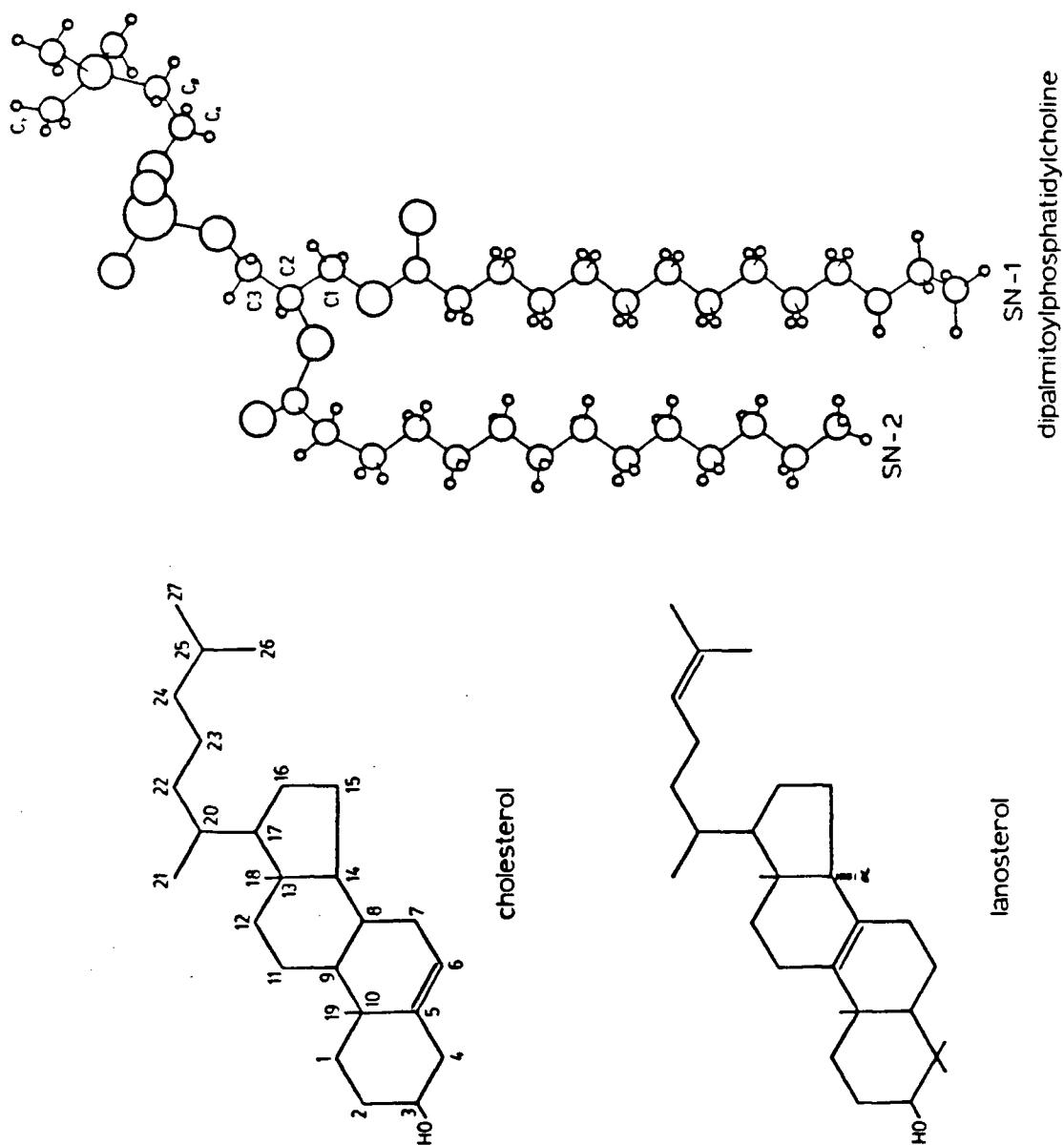
Figure 1. Molecular structures of cholesterol, lanosterol and dipalmitoylphosphatidylcholine.

the alpha face and the acyl chains [9,10].

Given that lanosterol is a precursor to cholesterol and the chief difference is the "bump" or protrusion from the alpha face, the comparative effects of these two sterols on membranes should indicate the importance of having the smooth face. Any differences should also provide a clue to why cholesterol is functionally superior.

Before reviewing some of the work done with these two sterols some necessary background material will be introduced.

A. BACKGROUND

Biological membranes contain a huge diversity of components. The majority of these components can be separated into two groups; proteins and lipids. Many studies have been undertaken to establish the contribution each component makes to the overall functioning of the the membrane.

The physiological roles of proteins include the transport of solutes across the membrane and enzymatic activities [12]. Membrane-associated proteins can be of two general types. If they are deeply embedded in the membrane they are known as integral proteins whereas peripheral proteins are associated with the hydrophilic regions of either the lipids or the integral proteins on the membrane surface.

Lipids provide a matrix for the proteins [13]. The reason for such a large number of different lipids is not

fully understood. Studies have indicated that membrane properties such as fluidity and permeability are dependent on lipid composition. Also, a variety of lipids may be necessary to satisfy packing requirements of proteins [14,15].

The roles of the integral proteins are likely to be influenced by lipid/protein interactions. These interactions have been discussed in detail elsewhere [16]. It will be of interest to compare the protein/lipid interactions to lipid/lipid interactions in light of the results of this work.

Interactions between lipids depend on the environment and the structures of the lipids involved. The response of phospholipids to changes in the environment vary with acyl chain length, degree of saturation and the type of headgroup [13]. This makes the interpretation of results from biological membranes both complicated and ambiguous. To remove some of this ambiguity, model membranes are often used. The actual model chosen depends on the nature of the study. A simple model may consist of a particular phospholipid dispersed in water. More sophisticated models may incorporate different lipids and/or proteins.

A typical model is for example an aqueous dispersion of the phospholipid DPPC (figure 1). Because of the amphipathic nature of phospholipids (i.e. they possess both hydrophilic and hydrophobic regions) and, in the case of DPPC, the basically cylindrical shape of the molecule, multilamellar

vesicles (MLVs) are the most energetically favorable structures formed when the phospholipids are dispersed in water [13]. These MLVs consist of concentric bilayers of lipids which resemble in structure the skins of an onion. Within the vesicles, the lipids are oriented such that the choline headgroups are positioned at the lipid/water interface while the acyl chains extend into the central region of the bilayer.

Sterols are also amphipathic, the hydroyl group represents the polar moiety and the cholestane ring and acyl chain the hydrophobic part.

The incorporation of sterols into phospholipid model membranes drastically modifies their physical properties, including permeability and phase behaviour. This must be brought about by some interaction between the sterol and the phospholipids. It is therefore important to determine the position of the cholesterol molecules with respect to the bilayers.

X-ray and neutron diffraction studies [17-19] on egg-PC/cholesterol mixtures indicate that the 3-β-hydroxyl group of cholesterol is positioned at the phospholipid/water interface in the vicinity of the acyl-ester linkage and the ring structure is oriented perpendicular to the bilayer and spans the first 8-10 methelene groups [7].

Since lanosterol also has a 3-β hydroxyl and is planar it is reasonable to assume that it is oriented in lipid bilayers in a similar manner to cholesterol. Further

evidence is provided by ^{2}H NMR studies using cholesterol-3ad_1 and lanosterol-3ad_1 in PC vesicles [20]. It is reasonable to ask whether the 14-a methyl group of lanosterol would perturb the acyl chain orientational order in the vicinity of the number 9 to 12 carbons in a manner different from cholesterol. The actual position closest to the 14-a methyl group is dependent on the temperature due to the change in length of the acyl chains with increasing temperature. The results of a study using fatty acid spin labels in lecithin (PC) vesicles with the two sterols showed that a significant perturbation in the fatty acid chains occurred in the vicinity of C_{12} [20].

Cholesterol exhibits a "condensation effect" [2] with some phospholipids. In other words, the mixing of cholesterol and the phospholipids is not ideal, so that the mean molecular area of a film of a cholesterol/phospholipid mixture is not a straightforward sum of the areas of the individual components. It is assumed that the cross sectional area of cholesterol is constant. The term condensation therefore refers to the reduction in cross-sectional area of the phospholipid per polar headgroup.

Two structural features of the sterol are essential for condensation to occur; the 3β-hydroxyl group and the planar conformation of the ring, both of which lanosterol possesses [22,23]. This effect probably arises from Van der Waals forces between the rigid tetracyclic nucleus and the acyl chains. The planarity of the alpha face promotes maximal interaction.

Physical studies with model membranes indicate that lanosterol does not condense phospholipid fatty chains [10].

B. PHASE BEHAVIOUR OF PHOSPHLIPID SYSTEMS

In a pure lipid system, the acyl chains of the lipids undergo freezing (or melting) at a characteristic transition temperature. Below this temperature the diffusion rate of the lipids is $<10^{-10} cm^2/s$ and the chains are oriented at an angle with respect to the bilayer (figure 2) [13]. This is known as the gel-phase of the lipids. The acyl chains melt as the temperature is raised through the transition. Above the transition temperature the lipids are in a liquid crystalline phase in which the orientational order of the chains is reduced considerably and in which the axis of symmetry for reorientational motions of the chain is perpendicular to the bilayer. The lipids diffuse laterally (i.e. in the plane of the bilayer) with a diffusion rate of $\simeq 10^{-8} cm^2/s$.

The change in state with increasing temperature involves changes in thermodynamic quantities such as entropy
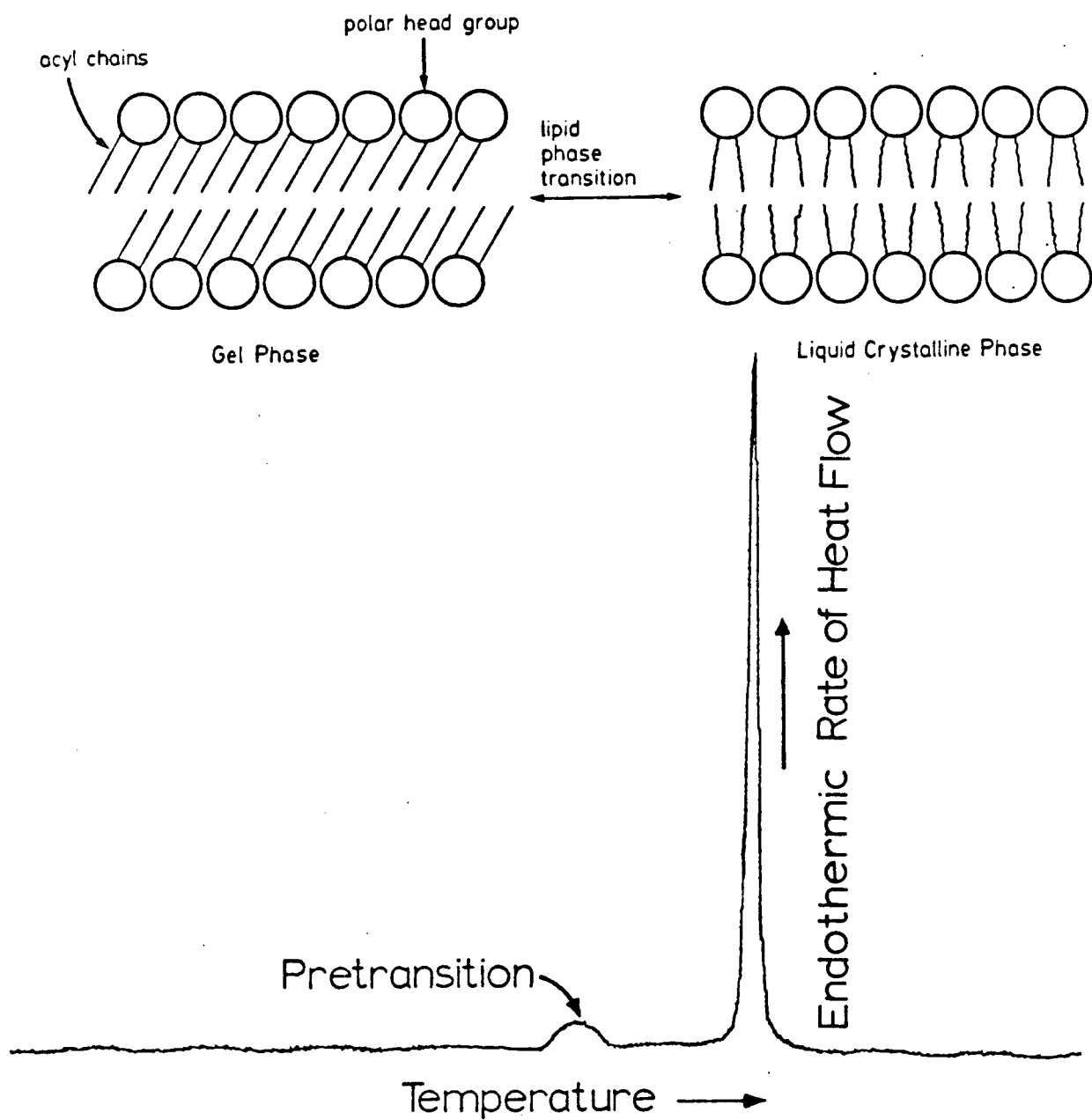
Figure 2. Lipid phase transitions. (A) Phase transition between gel and liquid crystalline states. (B) DSC trace of the calorimetric behaviour of DPPC-d31.

and enthalpy which can be interpreted quantitatively in terms of the observed decrease in order of the chains. In the gel-state the acyl chains are in a tilted all-trans configuration whereas in the liquid crystalline phase one or more gauche conformers may exist, resulting in increased interchain spacing. The conformation of the chain determines the angle the C-D bonds make with normal to the bilayer. This angle therefore provides some information on chain length, thickness and packing and can be determined from the order parameters of the deuteriums down the chain [21].

Also a quantitative measure of the extent of motional averaging is provided by the order parameters. For a C-D bond making an angle $\theta(t)$ at time t with the normal to the bilayer, the order parameter S_{CD} is defined as [24]

$$S_{CD} = \frac{1}{2} <3\cos^2(\theta(t))-1>$$
$$\text{1.1}$$

where the average is taken over all molecular conformations and motions. The methylene groups down the acyl chain at temperatures below the the chain melting temperature exhibit a more or less constant order parameter. Above the transition temperature, the degree of motion exhibited by deuterons on carbon positions 10-16+ increases monotonically with carbon number. The difference between the order parameters of deuterons closer to the headgroup (positions 2-9) is small which results in the characteristic plateau region in the order parameter profiles.

Differential Scanning Calorimetry can be used to follow the transition. The trace shown in figure 2 shows the heat absorbed by a pure phospholipid system as a function of temperature. The peak occurs at the transition temperature. For the pure lipid system the transition occurs over one degree as indicated by the width of the transition peak.

The behaviour of complex multi-lipid systems is more representative of biological membranes. Because of the number of different lipids the system does not undergo an isothermal phase transition. The system may exist in a mixed gel/liquid crystalline phase over a wide range of temperature. The motivation for studying phase behaviour is not only to model the thermodynamic behaviour of biological membranes (since at physiological temperatures membranes are in a liquid crystalline state) but to establish how the different lipids are interacting.

C. CHOLESTEROL/PHOSPHOLIPID SYSTEMS

Cholesterol is thought to decrease the order of lipids in their gel-phase and order them in their liquid-crystalline phase. The state of a model membrane containing DPPC and cholesterol therefore depends on both the sterol concentration and the temperature. Even for this ternary system this results in complex phase behaviour. The complexity is reflected in the phase diagram determined from DSC and Deuterium Nuclear Magnetic Resonance (^{2}H-NMR) (figure 3) [6].
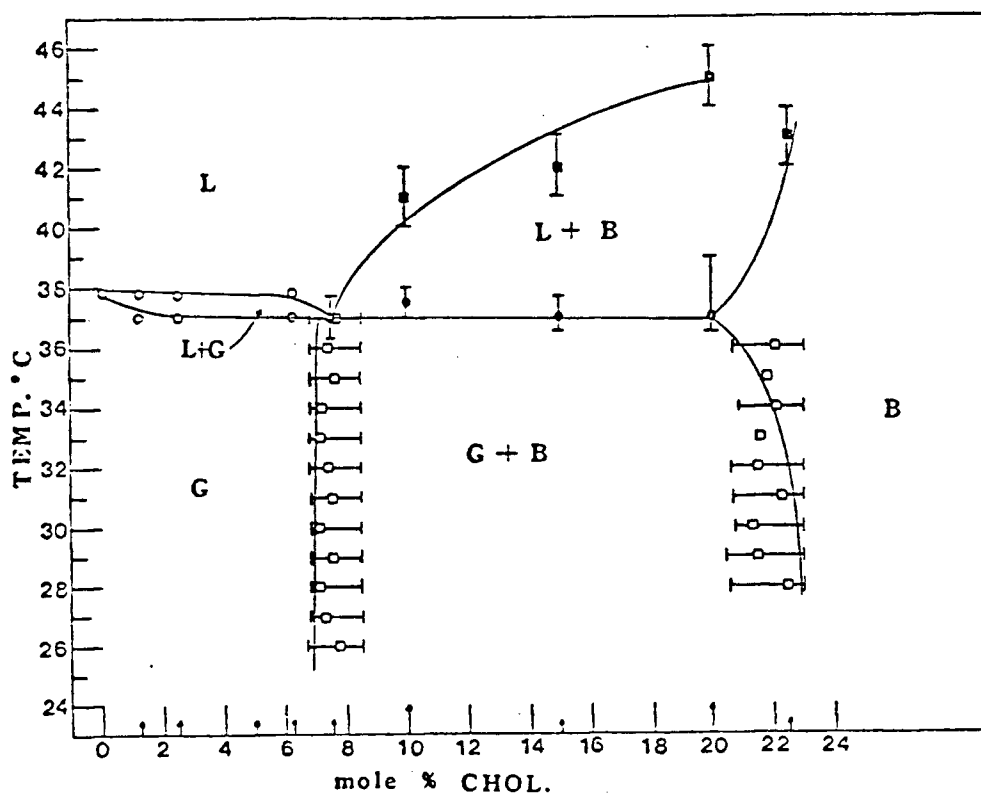
Figure 3. The temperature composition plot for DPPC-d_{62}/cholesterol. L is the liquid crystal fluid phase. G is the gel phase. L + β, G + β and L + G are two phase regions. o ^{2}H NMR data, ▫ Difference spectroscopy data, •■ DSC data. Diagram reproduced from [6].

DSC measurements indicate that the heat absorbed at the transition temperature decreases linearly with increasing cholesterol content [4,25], whereas the width of the transition peak broadens.

The condensation effect depends on the existence of an intact side chain and a 3β hydroxyl group. It is therefore interesting that these same requirements are needed for the broadening and possible elimination of the gel to liquid crystalline phase transition peak [2,4,26].

D. FLUIDITY

The term "fluidity" is often used to describe the physical state of membranes. Unfortunately it is a loosely defined parameter and has been used to describe different membrane properties. Fluidity is used in some cases to describe the orientational order of the molecules in a system while in other cases the definition of fluidity as being inversely related to microviscosity is used.

E. AIMS OF RESEARCH

Interactions between cholesterol and DPPC manifest themselves in many physically observable ways. Differential effects between lanosterol and cholesterol have been observed for membrane properties such as microviscosity, permeability etc. The aim of this research was to compare the influence of lanosterol on the chain dynamics of DPPC to cholesterol. Calorimetry was used to compare the

thermodynamic behaviour of the systems studied. Information about the orientational order was obtained using ^{2}H-NMR.

DPPC molecules with a perdeuterated sn-2 chain were used with the hope that if certain regions of the chain were perturbed by the bulky methyl group of lanosterol, the ^{2}H spectra would reflect this.

For further comparison between the systems the NMR relaxation time T_{2e} was also measured. Relaxation times are influenced by the amplitudes and timescales of the interactions between the deuterons and their environment.

II. NMR AND DSC THEORY

A. DEUTERIUM NUCLEAR MAGNETIC RESONANCE

A nucleus with spin I > 1/2 possesses an electric quadrupole moment (eQ). Electrons near the site of the nucleus give rise to an electric field gradient (EFG) which interacts with the electric quadrupole moment [27].

The EFG can be expressed as a traceless, symmetric second rank tensor $V_{\alpha\beta}$. By choosing a set of principal axes in relation to which $V_{\alpha\beta} = 0$ for $\alpha \neq \beta$, only two parameters are needed to describe $V_{\alpha\beta}$; the asymmetry parameter (η) and the field gradient (eq), where [27]

$$eq = V_{zz} \qquad\qquad 2.1$$

$$\eta = \frac{V_{xx} - V_{yy}}{V_{zz}} \qquad\qquad 2.2$$

In NMR in high magnetic fields, the direction of the external magnetic field is a natural choice for one of the axes in the laboratory frame. The principal axis of the EFG does not necessarily coincide with the laboratory frame. Transformation into the laboratory reference frame can be accomplished by the Wigner Rotation Matrix $D(\alpha\beta\gamma)$ operating on the EFG tensor when written in terms of a spherical basis [28]. The angles α, β and γ are the Euler angles defining the transformation.

The interaction between the EFG and the electric quadrupole moment lifts the degeneracy of the Zeeman energy levels. In a large magnetic field, H_0, the electric quadrupole interaction is much smaller than the Zeeman Interaction and can be treated as a first order perturbation on the Zeeman Interaction [27]. Since deuterium has spin I=1, there are two allowed transitions (m=-1 to m=0) and (m=0 to m=1). As a result of the quadrupole interaction these two transitions are characterized by different energies as illustrated in figure 4.

The spectrum from a system of equivalent ^{2}H-nuclei will be a doublet. The frequency separation of the two signals is [24]

$$\Delta \nu_Q = \frac{3}{4} \frac{e^2 qQ}{h} \ [(3\cos^2(\beta)-1)+\eta\cos(2a)\sin^2(\beta)] \qquad 2.3$$

where a, β are Euler angles specifying the orientation of the EFG tensor with respect to the magnetic field H_0.

The EFG tensor of the phospholipids is in a molecule fixed system. Motions modulate the orientation of the molecules with respect to H_0 and thus the observed quadrupole splitting will be

$$\Delta \nu_Q = \frac{3}{4} \frac{e^2 qQ}{h} <(3\cos^2(\beta)-1)+\eta\cos(2a)\sin^2(\beta)> \qquad 2.4$$

which is averaged over all motions that are fast compared to the static quadrupole coupling constant, e^2qQ/h ($\simeq$ 170 kHz for most C-D bonds).

A.
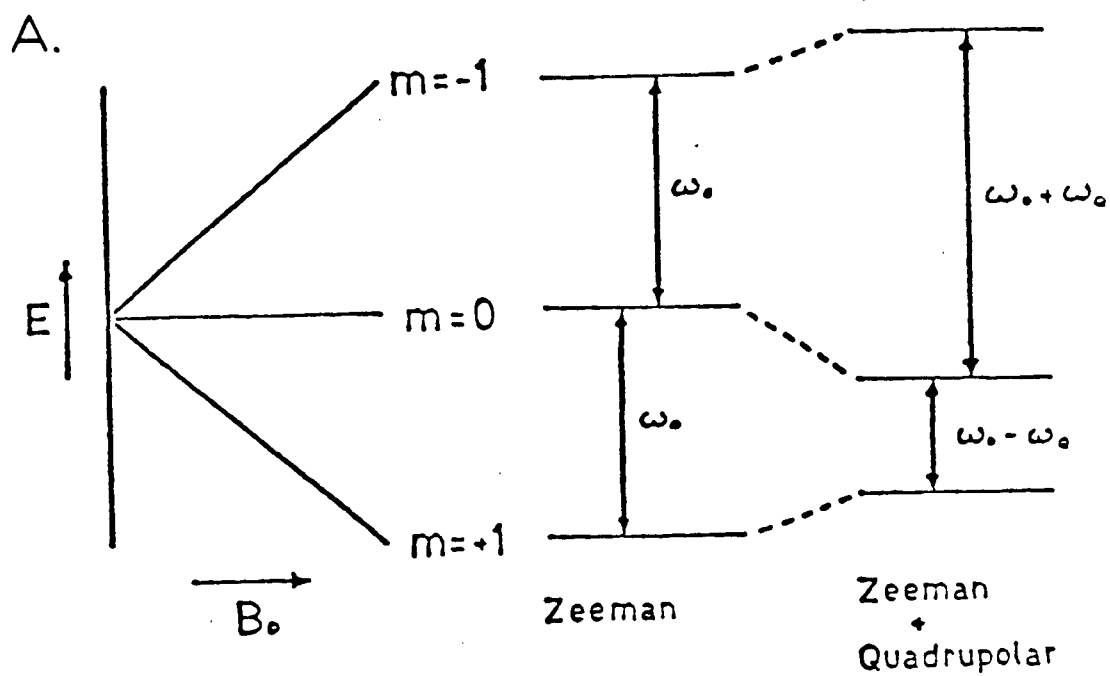
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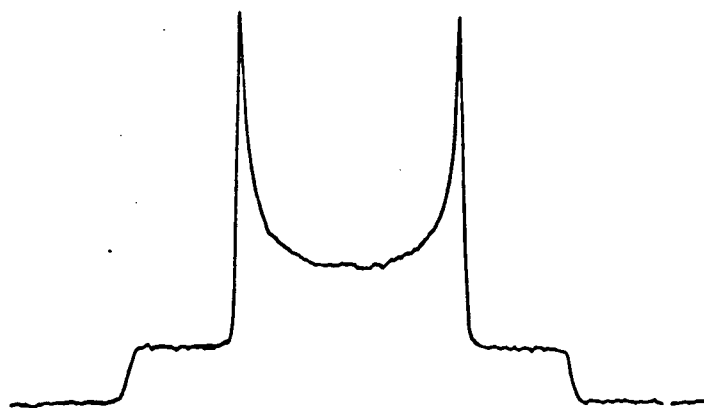
Frequency

Figure 4. Quadrupole Spectra. (A) Perturbed Zeeman energy levels. (B)A Typical Powder Pattern.

B. ORDER PARAMETERS

Lipids in the liquid crystalline state (figure 2) exhibit a high degree of axial symmetry around the normal to the bilayer (which is taken to be the z-direction). This leads to a zero asymetry parameter ($\eta = 0$). Rotations and flexing motions of the liquid crystalline phospholipids can also be charaterized by cylindrical symmetry [29] and therefore the angular component can be separated in two, giving

$$\Delta\nu_Q = \frac{3}{4} \frac{e^2 qQ}{h} (3\cos^2(\delta)-1)S_{CD} \qquad 2.5$$

where δ is the angle between the magnetic field and the normal to the bilayer [24]. The term S_{CD} is the order parameter for the C-D bond vector and is defined in equation 1.1.

C. THE ^{2}H POWDER PATTERN SPECTRUM, DEPAKING

Quadrupole NMR spectra that result from ^{2}H-lipid membranes are typical of the "powder patterns" seen for randomly oriented samples. The spectra are a superposition of doublets with quadrupole splittings from each of the randomly oriented domains. As a result of orientation dependent interactions, the contribution each domain makes to the lineshape is scaled [32]. If the width of the oriented lineshape as well as its splitting scale as $[3\cos^2\delta-1]/2$, a depaking procedure can be employed on the powder patterns resulting in the spectra that would be

obtained if the sample had been oriented with the field [30].

The number of domains perpendicular to H_0 (i.e. $\delta = 90$ in equation 2.5) far outnumber the domains parallel to the field [24], therefore the two most intense peaks in the powder pattern (figure 4(b)) correspond to the 90° edges. Powder patterns are depaked to give the oriented spectra which would be obtained for domains oriented at 90° to H_0 [30]. The expression for the splittings of the depaked lineshapes reduces to

$$\Delta \nu_Q = \frac{3}{4} \frac{e^2 qQ}{h} S_{CD} \qquad\qquad 2.6$$

Thus the order parameters can be easily determined from the splittings. Because of the dependence on bond angle the state of the membrane can be described by the order parameters.

D. FIRST MOMENT

In the conventional definition of the spectral moments all odd moments of a symmetric spectrum are zero [31]. In the case of spectra dominated by quadrupole interactions it is convenient to calculate moments of the half spectrum. For example the first moment of the half spectrum as defined below, is simply half

$$M_1 = \frac{\int_0^\infty f(\omega_0+\omega)\omega d\omega}{\int_0^\infty f(\omega_0+\omega)d\omega} \qquad\qquad 2.7$$

where ω_0 is the Larmor frequency and $f(\omega_0+\omega)$ is the lineshape at a frequency ω from ω_0.

In the powder patterns that result from phospholipids in which a single chain is perdeuterated there is a distribution of quadrupolar splittings ($\delta\nu_Q$) due to the variation in order along the chain. The first moment gives the average quadrupolar splitting;

$$M_1 = (4\pi/3\sqrt{3})<\delta\nu_Q> \qquad\qquad 2.8$$

Changes in the degree of order resulting from temperature variation or increasing sterol concentration can be followed by M_1. It is therefore a particularly useful parameter for examining the phase behaviour of model membranes.

E. <u>RELAXATION</u>

NMR relaxation times can give information on the "spectral density" of motions in model membrane systems [24,32]. These motions cause fluctuations in the spin dependent interactions.

The quadrupole echo resulting from the quadrupole echo pulse sequence (III.C) decays to zero as [exp($-t/T_{2e}$)] where T_{2e} is the spin-spin relaxation time. T_{2e} is sensitive to

slow motions such as translational diffusion and chain mobility ($\tau > 10^{-7}$s).

Spin lattice relaxation measurements (T_1) provide information on motions having correlation times between 10^{-9}s to 10^{-8}s approximately [16,32] i.e. of the order of the larmor frequency.

It has been proposed [32] that collective motions of hydocarbon chains give rise to a distribution of correlation times extending to quite long values. Such a distribution of correlation times would lead to short T_{2e} values.

Previous studies with perdeuterated phospholipid systems have found that relaxation curves vary exponentially with short τ values where τ is the time separating the two 90°pulses (III.C) [33]. The initial decay rate provides an estimate of the average T_{2e} for the many spins in the system.

F. DIFFERENTIAL SCANNING CALORIMETERY

Differential scanning calorimetery measures the difference in heat needed to maintain a very small but definite temperature difference between two samples as the temperature is varied [34]. One sample is usually inert (i.e. does not undergo a phase transition in the temperature range of interest) and is used as a reference.

The DSC trace shown previously (figure 2) shows a variation in the excess specific heat (C_{ex}) with temperature. The enthalpy for the transition between gel

(T < 39°C) and liquid crystalline (T > 39°C) is [34]

$$\Delta H_{cal} = M_L \int_{T_1}^{T_2} C_{ex} \, dT \qquad 2.9$$

where M_2 is the molecular weight of the lipid DPPC-d31 and T_1 and T_2 are the endpoints of the transition peak. This peak can also be characterized by its halfwidth which indicates the temperature range of the transition.

As the chains melt from the gel configuration there is a change in the free energy, ΔG. The enthalpy change for the transition is

$$\Delta H = \frac{\partial(\Delta G/T)}{\partial(1/T)} \qquad 2.10$$

from $\Delta G = \Delta H - T\Delta S$ (for constant pressure) where ΔH can be identified with the integrated enthalpy of the DSC trace.

The enthalpy per mole of DPPC, $\Delta H/n_1$, varies linearly with the concentration of cholesterol incorporated. The slope of a plot of $\Delta H/n_1$ vs % cholesterol has been interpreted as indicating the fraction of lipid not participating in the transition [35].

III. MATERIALS AND METHODS

A. NMR SAMPLE PREPARATION

The phospholipid sn-2 [$^2H_{31}$] dipalmitoyl phosphatidylcholine (DPPC-d31) was kindly provided by R. Cushley. The synthesis of this lipid is outlined in [36]. Using TLC, the purity was found to be greater than 98%. DPPC (99+% purity), cholesterol (98%+ purity), and lanosterol (80% purity) were obtained from Sigma Chemical Co.

Stock solutions of the two sterols were prepared in chloroform and appropriate volumes were added to the phospholipid samples to reach the desired sterol concentration. Typically, the dry weight of the samples was between 50-60 mg.

The sample was gently rotated in a warm bath while the solvent was pumped off, leaving a thin homogeneous film on the flask. To remove the remaining solvent the samples were dried under high vacuum for a period of at least 6 hours.

Aqueous dispersion of the lipids were formed by adding excess buffer (50mM potassium phosphate pH 7) to the films. These dispersions were subjected to vigorous mixing (vortexing) to form multilamellar vesicles. Freeze/thaw cycles were repeated at least three times to disrupt the vesicles, causing water to be distributed evenly throughout the vesicles into vesicles of similar sizes thereby eliminating some of the sample inhomogeneity.

The dispersions were centrifuged and the resulting pellets were separated from the supernatant and placed into 6mm wide, 1.5cm long test tubes. Deuterium depleted buffer (again 50mM potassium phosphate) was added in excess, usually about 60-100μl. Again the freeze/thaw/vortexing cycles were repeated. All samples were used within 2 days of preparation and all NMR experiments were completed within 4 days. TLC was performed before and after the experiments to ensure no degradation had occurred during the experiment. None was observed. The eluting solvent for the TLC was $CHCl_3$:MeOH:25%NH_3:H_2O in the ratio 900:540:57:53.

B. NMR MEASUREMENTS

All NMR measurements were performed on a locally constructed 35 MHz deuterium spectrometer operating on resonance. Quadrupole echos were obtained by repeating the pulse cycle consisting of four pairs of pulses $[90_x-\tau-90_y]$, $[90_{-x}-\tau-90_y]$ $[90_x-\tau-90_{-y}]$, $[90_{-x}-\tau-90_{-y}]$ where the echos resulting from each pair of pulses were alternately added to and subtracted from the computer memory. The length of the 90 degree pulse was $\simeq 3\mu$sec and $\tau=40\mu$sec. This sequence was repeated at a rate of 2.5 s^{-1}. Since the T_1 of these samples was on the order of 35 msec this recycle time was sufficiently long to allow the deuterons to equilibrate along the field (with the exception of the methyl deuterons which have a much longer T_1) [30]. Quadrature detection was used and the echo signal both in and out of phase with the applied r.f. pulse were

collected. The phases were adjusted so that the signal in the out of phase channel was effectively zero.

Above the transition temperature of the phospholipid, a dwell time of 5μsec was used whereas a dwell time of 2μsec was used for lipids below the transition temperature. Each spectrum was obtained from the accumulation of 40,000 scans.

When necessary, the signal was shifted using an interpolation technique [37] to ensure that the echo was symmetrical about $t=2\tau$. The signal was transformed into the frequency domain using a fast Fourier Transform. Spectral moments were calculated from the spectra in the frequency domain and oriented spectra were obtained by depaking the powder patterns.

The average T_{2e} of the deuterons on the sn-2 chain was measured using the quadrupole echo sequence with varying τ values.

The temperature was regulated by a Bruker Temperature Controller. The heating device consisted of an oven and the temperature was measured using a thermocouple. At each temperature change the system was allowed to equilibrate for an hour.

C. CALORIMETRY

Differential Scanning Calorimetry (DSC) measurements were performed on an MC-1 Ultra Sensitive Scanning Calorimeter MicroCal Inc. The samples were prepared in a similar manner to the NMR samples except DPPC not DPPC-d31 was used. DSC

traces of DPPC-d31 were shown to exhibit similar calorimetric behaviour to DPPC, with the only observable difference being the 2 degree reduction in transition temperature. Each sample contained 5 mg of DPPC and the appropriate amounts of sterol to make 0,5,10,15,20 and 25 molar percent concentrations. The lipids were dispersed in .7 ml of buffer (again 50mM potassium phosphate) and the calorimetric behaviour of these samples was compared to a reference cell containing .7 ml of buffer.

All DSC traces were run at a scan rate of 14 degrees/hour. The enthalpy of transition was calculated from the area under each trace.

A. INTRODUCTION

Aqueous dispersions of sn-2 substituted DPPC-d31 with various concentrations of either lanosterol or cholesterol were studied using ^{2}H-NMR techniques. ^{2}H-NMR is sensitive to the behaviour of the deuterated chains. Thus these chains can be viewed as probes into the interior of the membranes, providing information about the order and dynamics of the bilayer.

B. PURE DPPC-D31 AT 44°C

A typical spectrum for these systems is shown in figure 5(a). The system contained pure DPPC-d31 vesicles in excess buffer and had been allowed to equilibrate at 44°C for an hour. The central peak, (at $\omega=0$), is due to the natural abundance of ^{2}H in water and was minimized by using a deuterium depleted buffer.

The carbon positions from the phospholipid head down to the terminal methyl group exhibit successively more motional averaging. Thus the peaks corresponding to the terminal methyl groups have the smallest splitting and are centred around ±3 kHz, while the deuterons positioned closer to the lipid/water interface (with the exception of the deuterons on the number two carbon) have increasingly larger splittings. Taking into account this "flexibility gradient", each resolved peak can be assigned a carbon position (or
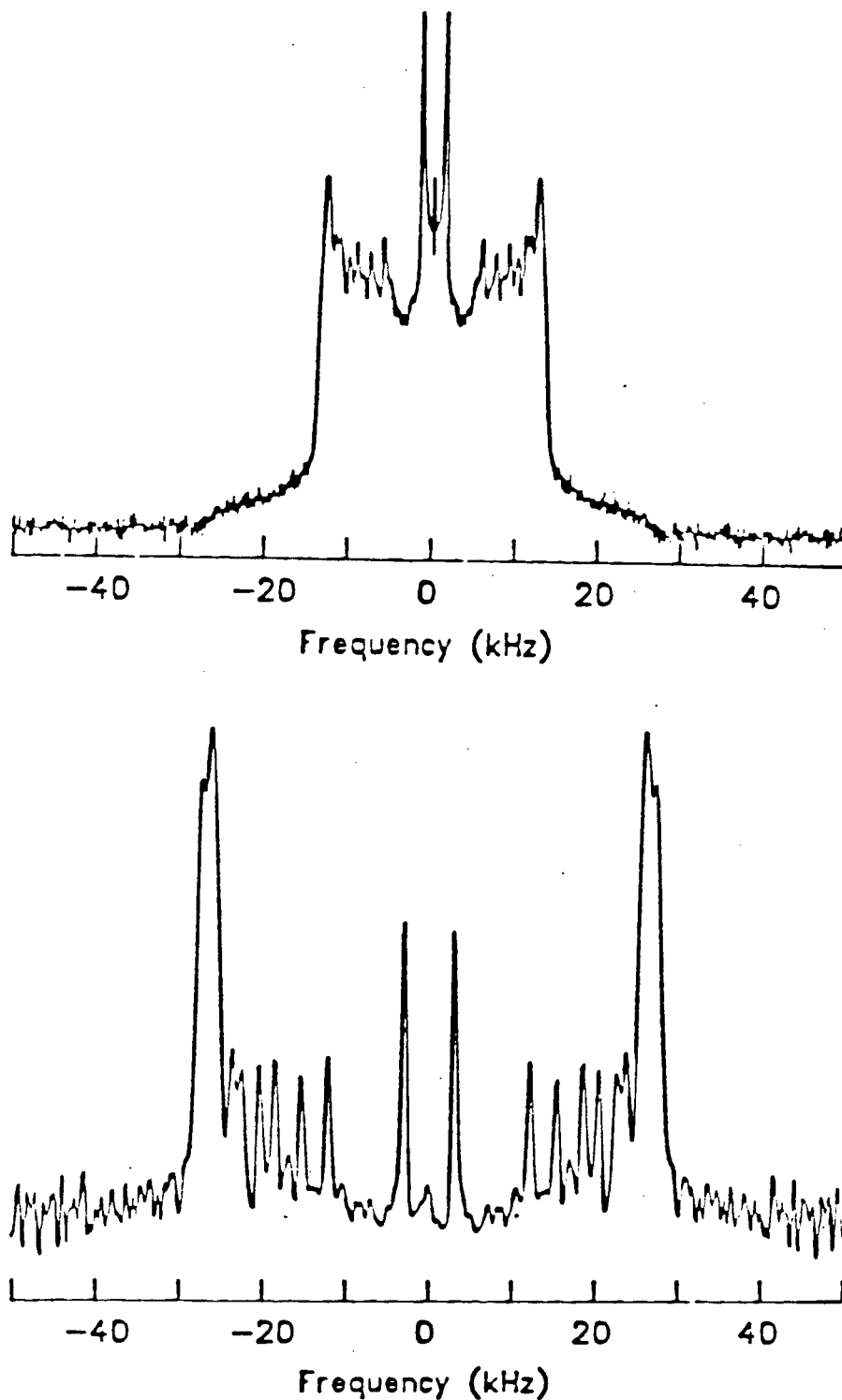
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Figure 5. ^2H NMR Spectra. (A) DPPC-d31 powder pattern taken at 44°C. (B) Depaked version of the above spectrum exhibiting 8 resolved peaks. The peak assignments indicate the carbon position the deuterons are bonded to.

carbon positions) on the chain. This can be achieved at least approximately by integrating the area under the depaked spectrum (figure 5(b)). Contributions to the composite lineshape are made by the methylene deuterons on carbon positions 2-15 and the methyl peaks on position 16. The total area at frequency splittings greater than that of the methyl peak (labeled 16 in figure 5(b)) can be partitioned among the 28 methylene deuterons. The area under each peak provides an estimate of the number of deuterons contributing to that peak. Thus each peak can be assigned a carbon position as shown in figure 5(b).

The recycle time of the experiment was shorter than the T_1 of the terminal methyl group so the area under the methyl peak does not scale with the number of deuterons [30]. It should also be mentioned that the deuterons on the alpha position carbon are inequivalent due to a bend in the chain at this point [38]. The small peaks with splittings of around 11 kHz and 17 kHz are probably attributable to these alpha position deuterons but the signal to noise ratio is not good enough to make any firm conclusions. A similar procedure was followed by Pauls *et al* [33] in their analysis of DPPC-d62 but the displacement of the ^2H NMR splittings associated with the sn-1 and sn-2 chains relative to each other caused the splittings in the DPPC-d62 spectrum to be less definitive than those made for DPPC-d31.These

splittings can be found in figure 13 and table 1.

Table 1. Quadrupole Splittings (kHz.)†

Carbon No.	DPPC-d31	5% Chol.
3-9	26.2	31.0
10	23.5	31.0
11	22.5	25.0
12	20.3	23.0
13	18.6	19.3
14	15.4	15.2
15	12.1	12.9
16	2.9	3.9

†All errors for the splittings are ± 0.1 kHz.

Two comments can be made on the assignment of the peaks in the depaked spectrum. Firstly, the difference in the splittings for positions 3-9 were not resolvable. These lineshapes are superimposed, forming what is known as the "plateau" region of the spectrum. Secondly, since the assignment of these peaks is subject to some ambiguity, further work with specifically labelled lipids would be necessary to establish unqualified assignments.

C. LANOSTEROL:DPPC-D31 SYSTEMS

In order to compare how the incorporation of lanosterol and cholesterol into DPPC-d31 bilayers influences the DPPC-d31 ^{2}H NMR spectrum, spectra were obtained for aqueous

dispersions of DPPC-d31 samples containing 0%, 5%, 10% and 15% molar percent lanosterol and 5%, 10% and 15% molar percent cholesterol as a function of temperature. The relaxation time T_{2e} was also measured to provide further insight into the dynamics of the lipids.

The spectra resulting from pure DPPC-d31 vesicles can be seen in figure 6 . As has already been discussed, this system exhibits axially symmetric spectra with 8 clearly resolvable peaks above the phase transition. It can also be seen that the phase transition is sharp, i.e. it occurs over a temperature range of less than two degrees and there is no observable overlapping of phases at temperatures either side of the transition (36°C,38°C).

Below the phase transition temperature, T_m, a broad gel-type spectrum is obtained. The shape is characteristic of the asymmetric spectra obtained for specifically deuterated lipids in the gel phase. Except for the methyl peaks it is essentially featureless. As the temperature is increased, the width of the powder pattern decreases, indicating that the lipids are becoming more mobile. These observations will be quantified to some extent when the first moments are presented.

The addition of 5 molar percent lanosterol reduces the resolution of the quadrupolar peaks above T_m, and increases the quadrupole splittings of the peaks that remain resolved, (figure 7). Only 5 peaks are clearly resolved in the depaked spectra (figure 16) so it was not possible to make

45 °C

41 °C

38 °C

36 °C

29 °C
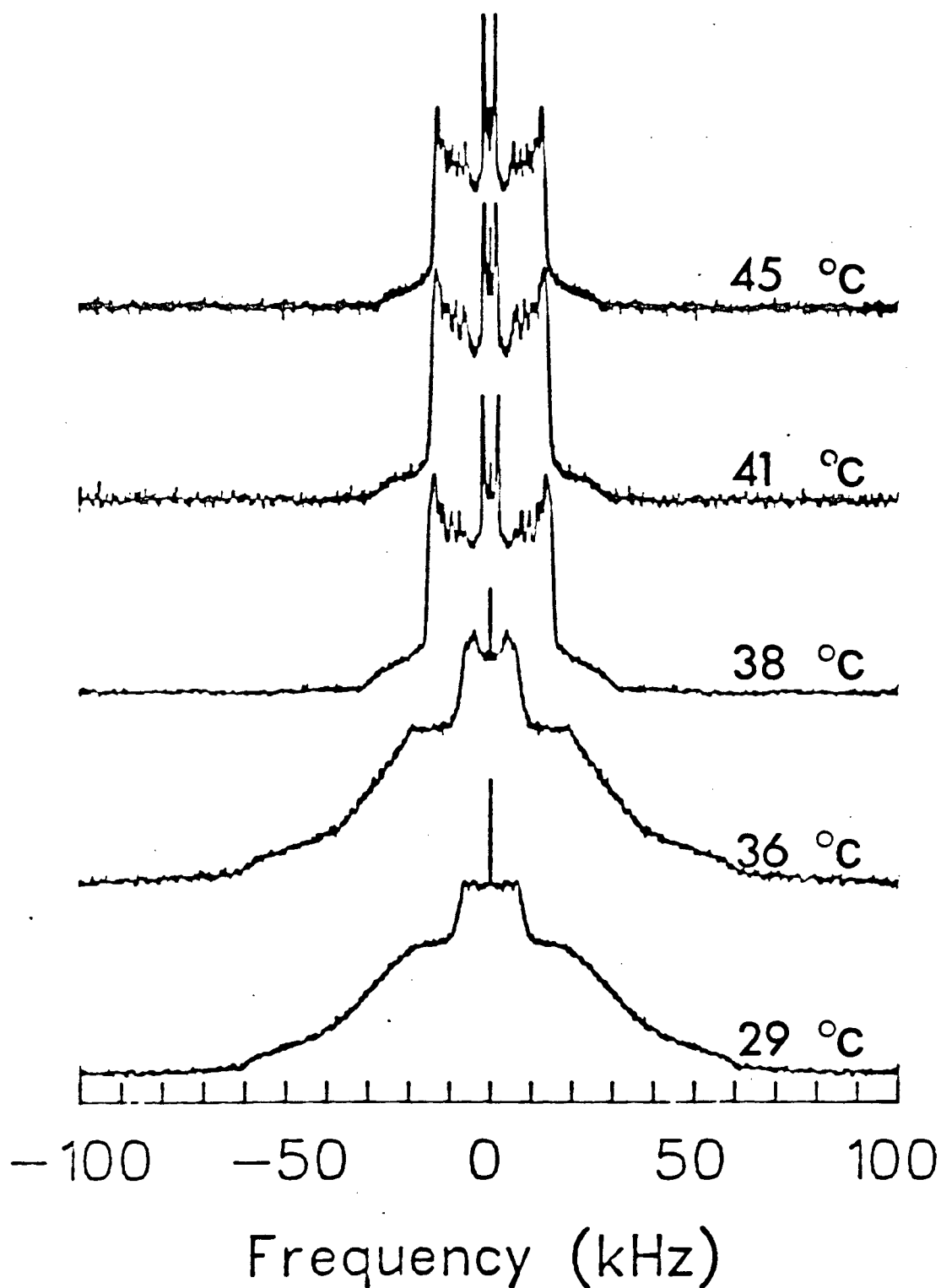
−100   −50   0   50   100

Frequency (kHz)

Figure 6. Comparison of the powder pattern spectra for pure DPPC-D31 measured over a range of temperatures.
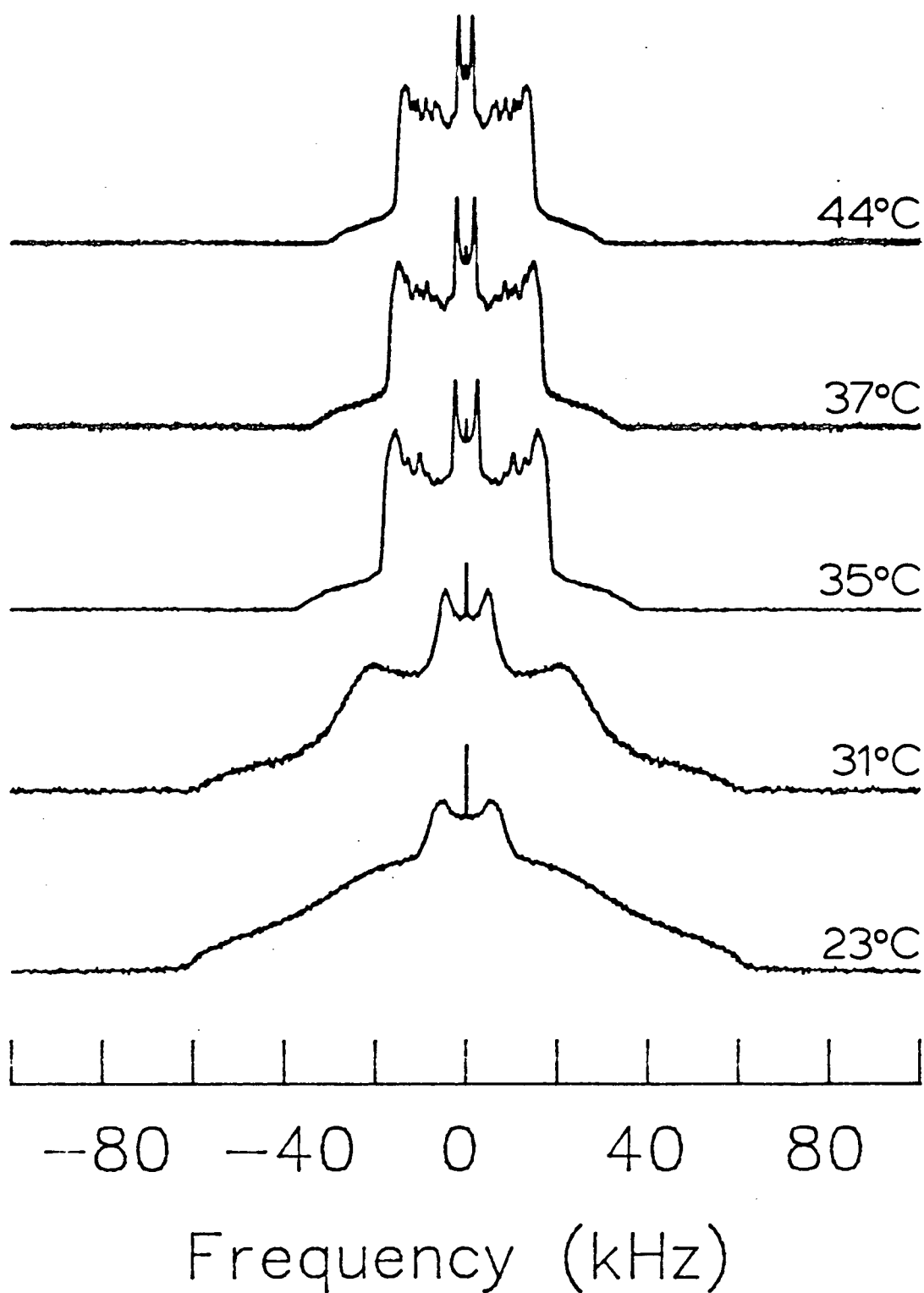
Figure 7. Comparison of the powder pattern spectra for 5% lanosterol/DPPC-d31 measured over a range of temperatures.

unambiguous peak assignments. The increased quadrupole splittings of the spectra is qualitatively consistent with the behaviour reported for cholesterol [6,7].

At 44°C, the T_{2e} relaxation time (table 2) is similar to the corresponding T_{2e} for pure DPPC-d31 at this temperature.

Table 2. T_{2e} Relaxation Times at 44°C.

Sterol Concentration	$T_{2e}(\mu sec)$
0% sterol	400
5% lanosterol	450
10% lanosterol	700
15% lanosterol	600
5% cholesterol	800
10% cholesterol	850
15% cholesterol	600

Thus the decreased resolution can not be explained by homogeneous line broadening.

Further, the transition occurs over a range of temperatures and between 34°C and 41°C the spectra contain contributions from both the gel and the liquid crystalline phases. Little attempt will be made to describe this mixed phase region other than by making the observation that it exists and later (in the discussion) by estimating the fractions of gel and liquid crystalline phases using the first moments.

With the lanosterol concentration increased to 10%, the resolution of the spectra above the phase transition is further reduced (figure 8). Only 4 peaks were resolved in the depaked spectra. The quadrupole splittings increase and the phase transition now extends over more than 10 degrees.

Similarly, the addition of 15% lanosterol continues the trend started by the addition of the smaller concentrations (figure 9). Over the entire temperature range it becomes difficult to say whether the system is in a single or mixed phase. The spectra remain axially symmetric to below 27°C.

This mixing of the phases can be further characterized by the first moment. As was discussed in the theory section, M_1 gives an estimate of the average order parameters or the average splittings. Although the definition of M_1 given in the theory section involved an integral, in practice the sum

$$\frac{\displaystyle\sum_{\omega=0}^{\omega_m} f(\omega_0+\omega)\omega}{\displaystyle\sum_{\omega=0}^{\omega_m} f(\omega_0+\omega)} \qquad\qquad 4.1$$

is evaluated, where $f(\omega)$ is the intensity at a frequency ω from the larmor frequency and ω_m is the frequency chosen to be in the baseline of the spectrum. The first moments of the spectra presented thus far are shown in figure 10. An initial comment on the transition can be made. For pure DPPC-d31 the difference between M_1 above the phase transition (44°C) and below the phase transition (23°C) is

Figure 8. Comparison of the powder pattern spectra for 10% lansterol/DPPC-d31 measured over a range of temperatures.

46 °C

44 °C

38 °C

36 °C

34 °C

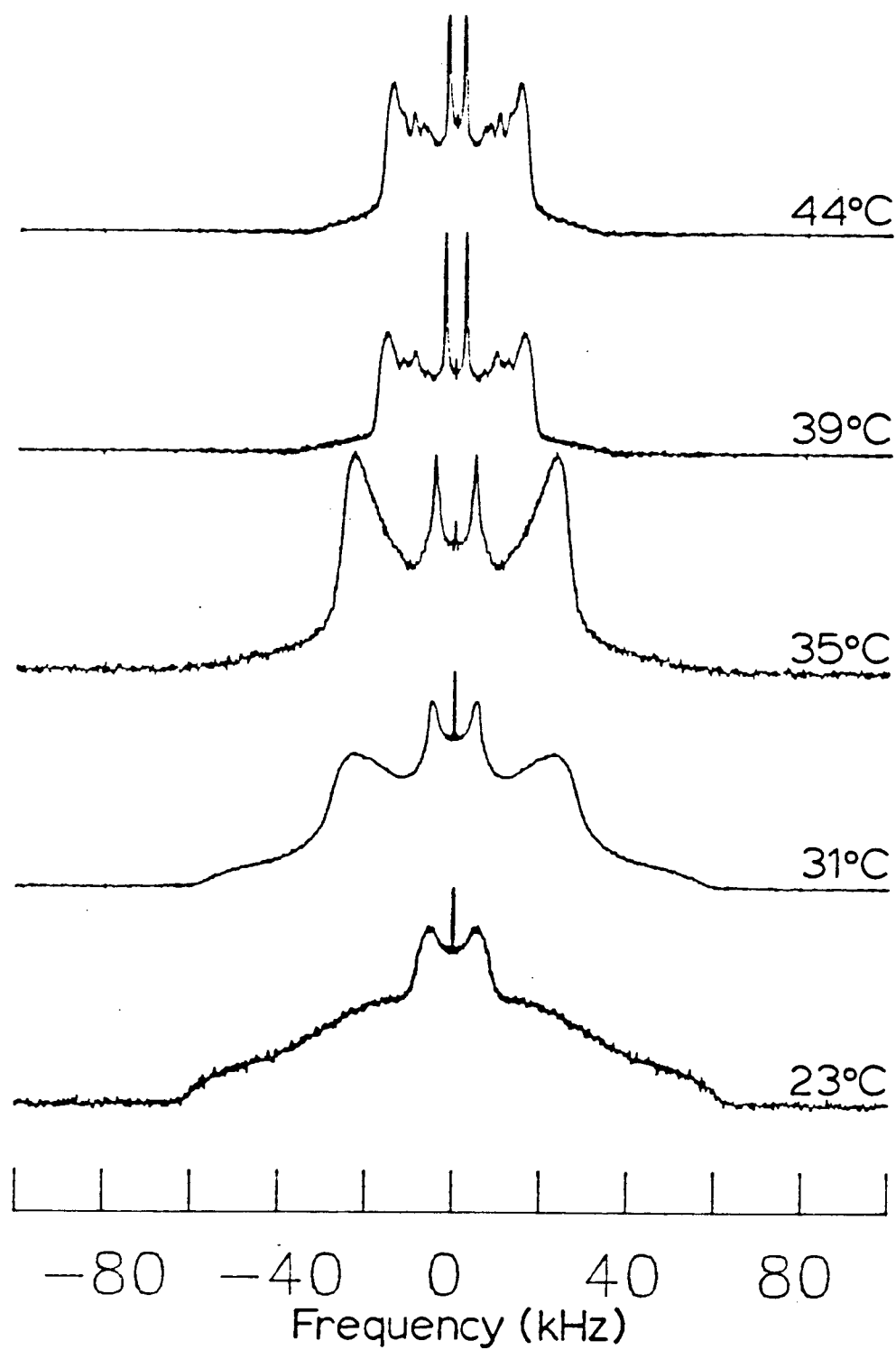27 °C

23 °C

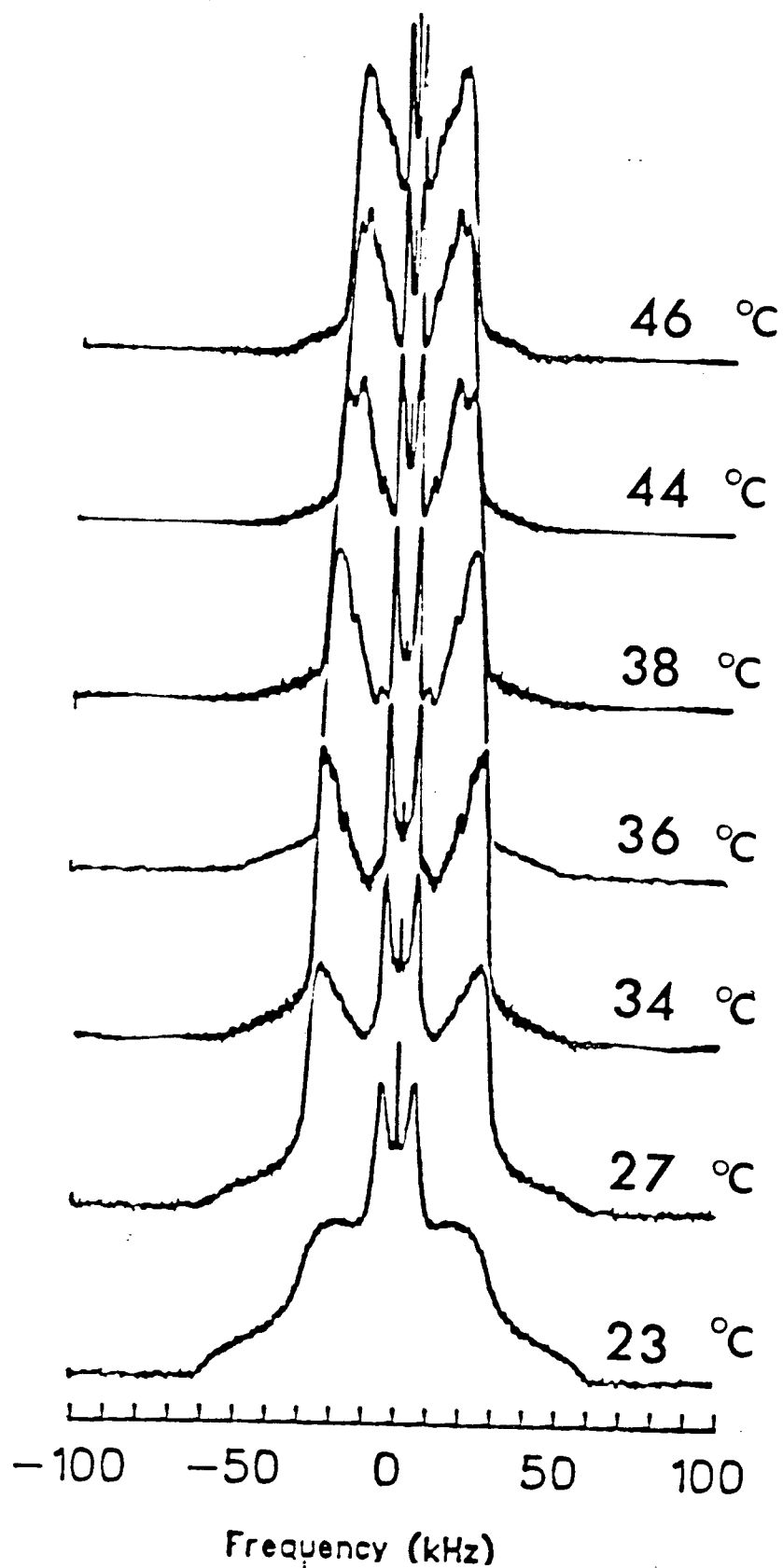−100   −50   0   50   100

Frequency (kHz)

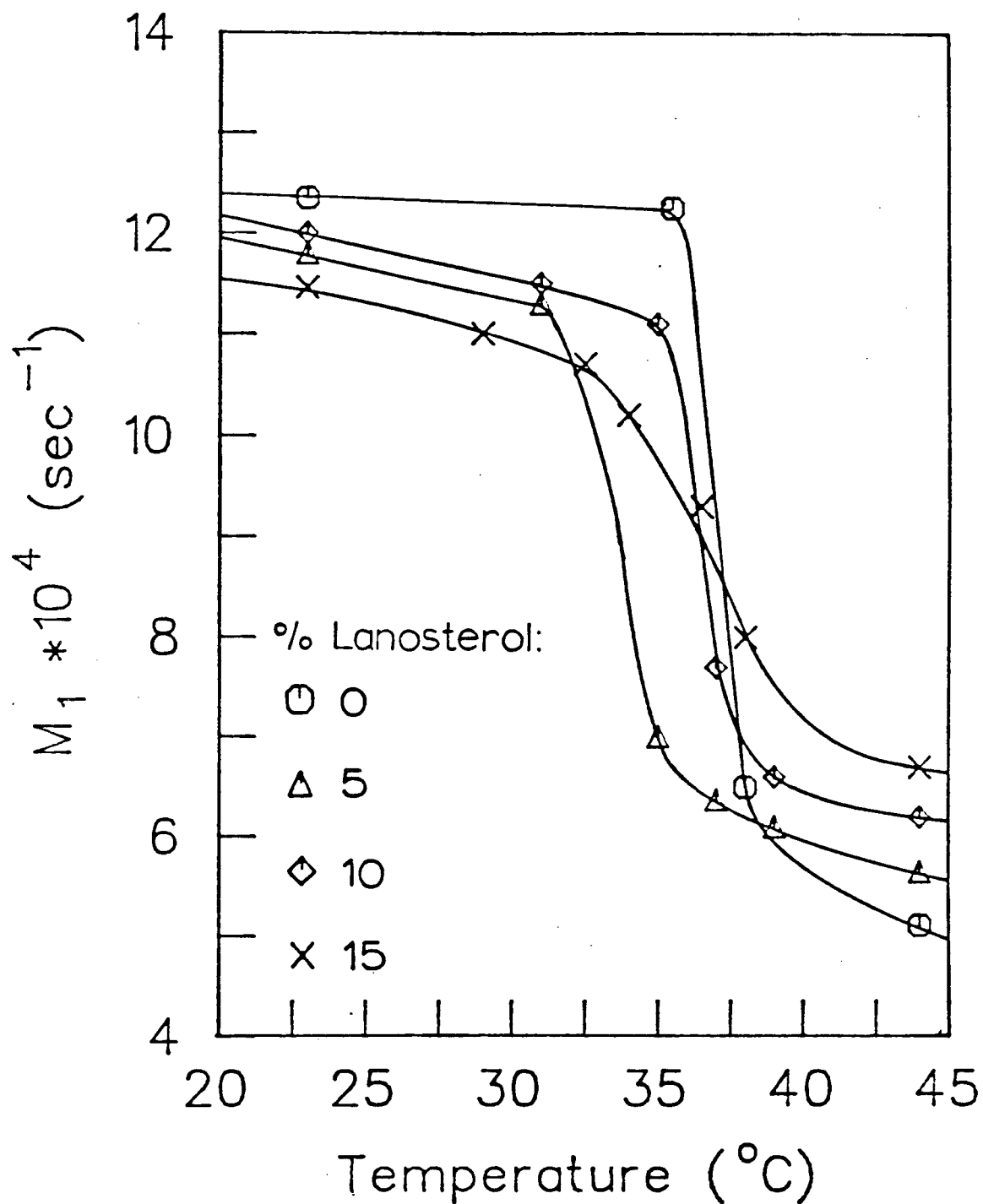Figure 9. Comparison of the powder pattern spectra for 15% lansterol/DPPC-d31 measured over a range of temperatures.

Figure 10. Variation of the first moment, M_1, with temperature for 0, 5, 10 and 15 percent lanosterol concentrations.

$7.5*10^{+4}$ s^{-1}. However, for 5% lanosterol this decreases to $6.4*10^{+4}$ s^{-1} further decreases to $5.8*10^{+4}$ s^{-1} and $4.8*10^{+4}$ s^{-1} with the addition of more lanosterol to concentrations of 10% and 15%. In fact at a given temperature above T_m , M_1 increases with sterol concentration while below T_m, M_1 decreases with sterol concentration. The behaviour of the moments supports the conclusions already reached about the phase transition. Without the incorporation of sterol, the transition from gel to liquid crysalline was sharp while with the addition of 15 molar percent lanosterol, a very gradual change in the moments occurred.

D. CHOLESTEROL:DPPC-D31 SYSTEMS

The powder patterns resulting from the addition of 5% cholesterol are similar to the corresponding 5% lanosterol spectra (figure 11). The quadrupole splittings are increased to a slightly greater extent than the lanosterol spectra (as will be seen from the plots of M_1) (figure 16). Seven peaks are clearly resolved and can be assigned carbon positions on the chain. Position 10 is no longer distinguishable from the plateau peak.

The quadrupolar relaxation time T_{2e} has increased dramatically (table 2) from 400 μsec (pure DPPC-d31) to 800μsec (5% cholesterol). Since the width at half maximum for a pure homogeneously broadened system is $1/\pi T_{2e}$ a comparison of the widths of the peaks in the depaked spectrum to $1/\pi T_{2e}$ shows there is an inhomogeneous
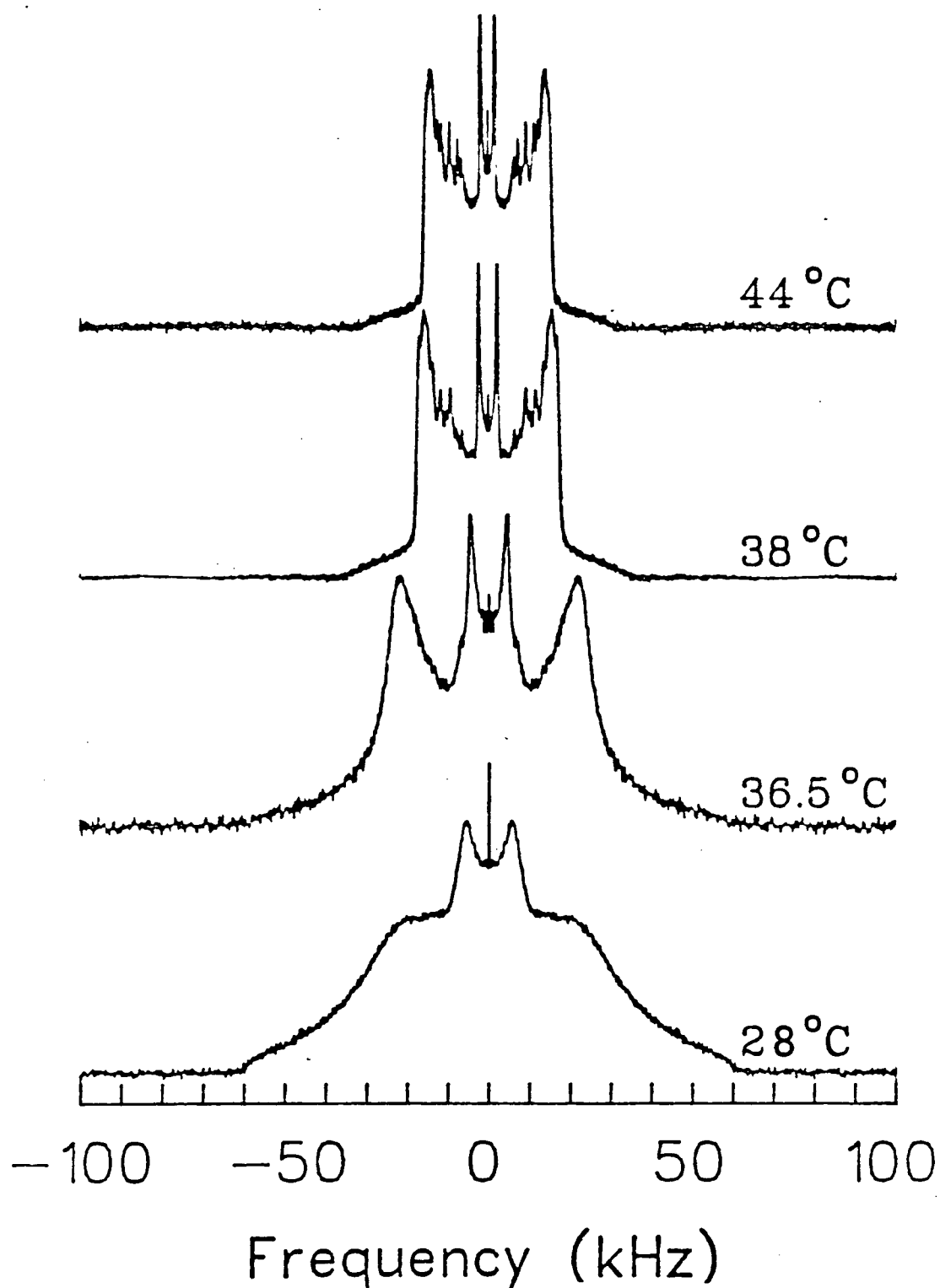
Figure 11. Comparison of the powder pattern spectra for 5%
cholesterol/DPPC-d31 measured over a range of temperatures.

contribution to the linewidth. For DPPC-d31, $1/\pi T_{2e} = .7$ kHz whereas the mean half width of the peaks is around 1 kHz. In the presence of 5% cholesterol, $1/\pi T_{2e}$ is .35 kHz while the average halfwidth of the peaks is 1.3 kHz., thirty percent larger than the average DPPC-d31 spectral peak width.

The transition from gel to liquid crystalline phases occurs over a wider range of temperatures than the pure DPPC-d31 transition as has previously been observed by others [6] and at 36.5°C the spectrum exhibits what has been described as the "liquid gel phase" [39]. At 28°C the system appears to be in the gel phase.

The results found for the 10% cholesterol sample were consistent with the 5% cholesterol data though the peak resolution had diminished somewhat (figure 12). Increasing the cholesterol concentration to 15% led to a significant decrease in the peak resolution (figure 13). Again the separations of the quadrupole splittings have increased. Only 4 peaks are resolved in the central region.

The behaviour of M_1 for 0%, 5%, 10% and 15% cholesterol is shown in figure 14. Very little difference exists between the first moments of cholesterol and lanosterol at comparable concentrations and temperatures. In both cases the transition region broadens with increasing sterol concentration. Within this transition region the lipids exist in a mixed phase.

Although the moments provide information about the average splittings, it would be of interest to see how the
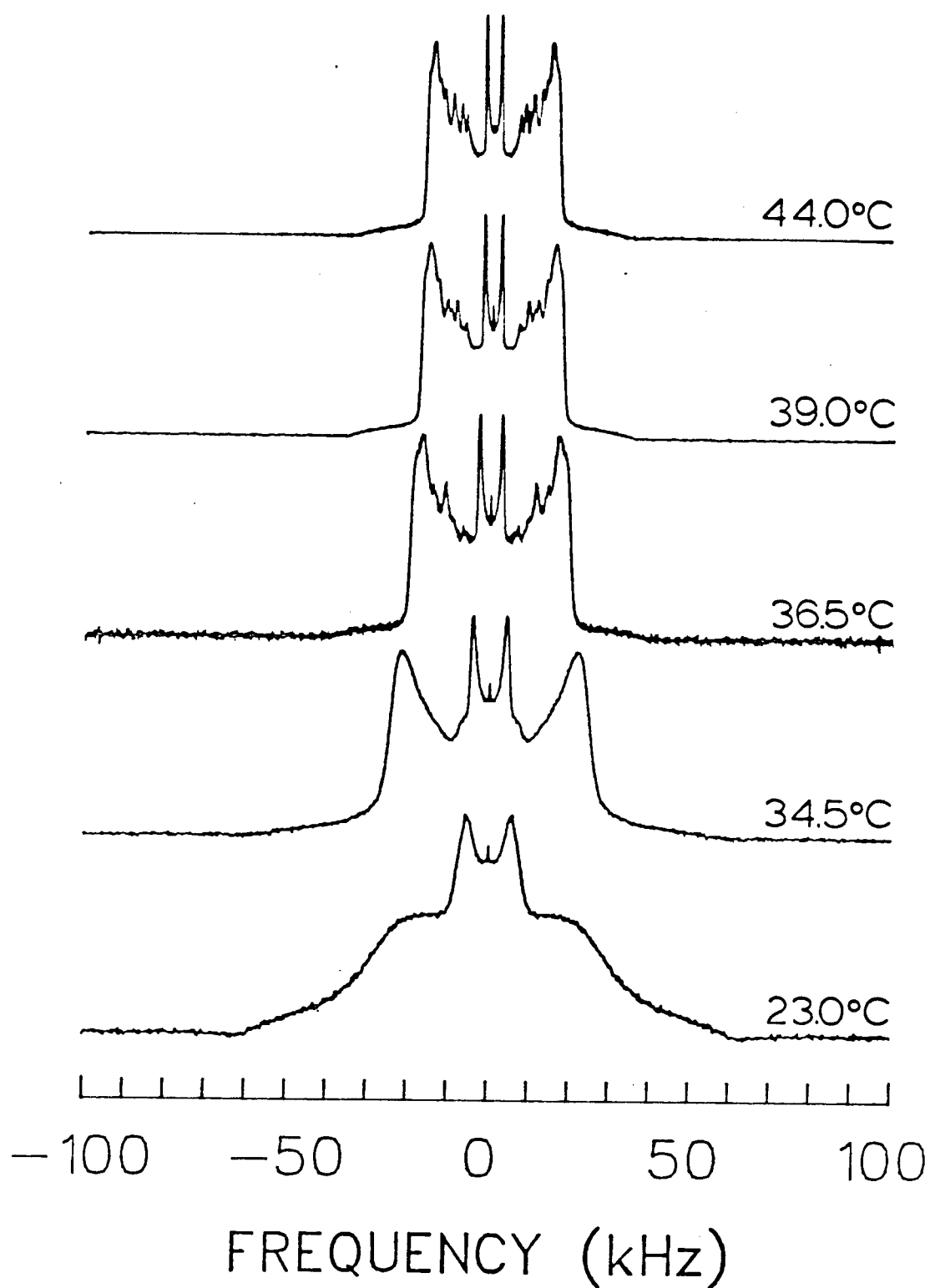
Figure 12. Comparison of the powder pattern spectra for 10% cholesterol/DPPC-d31 measured over a range of temperatures.
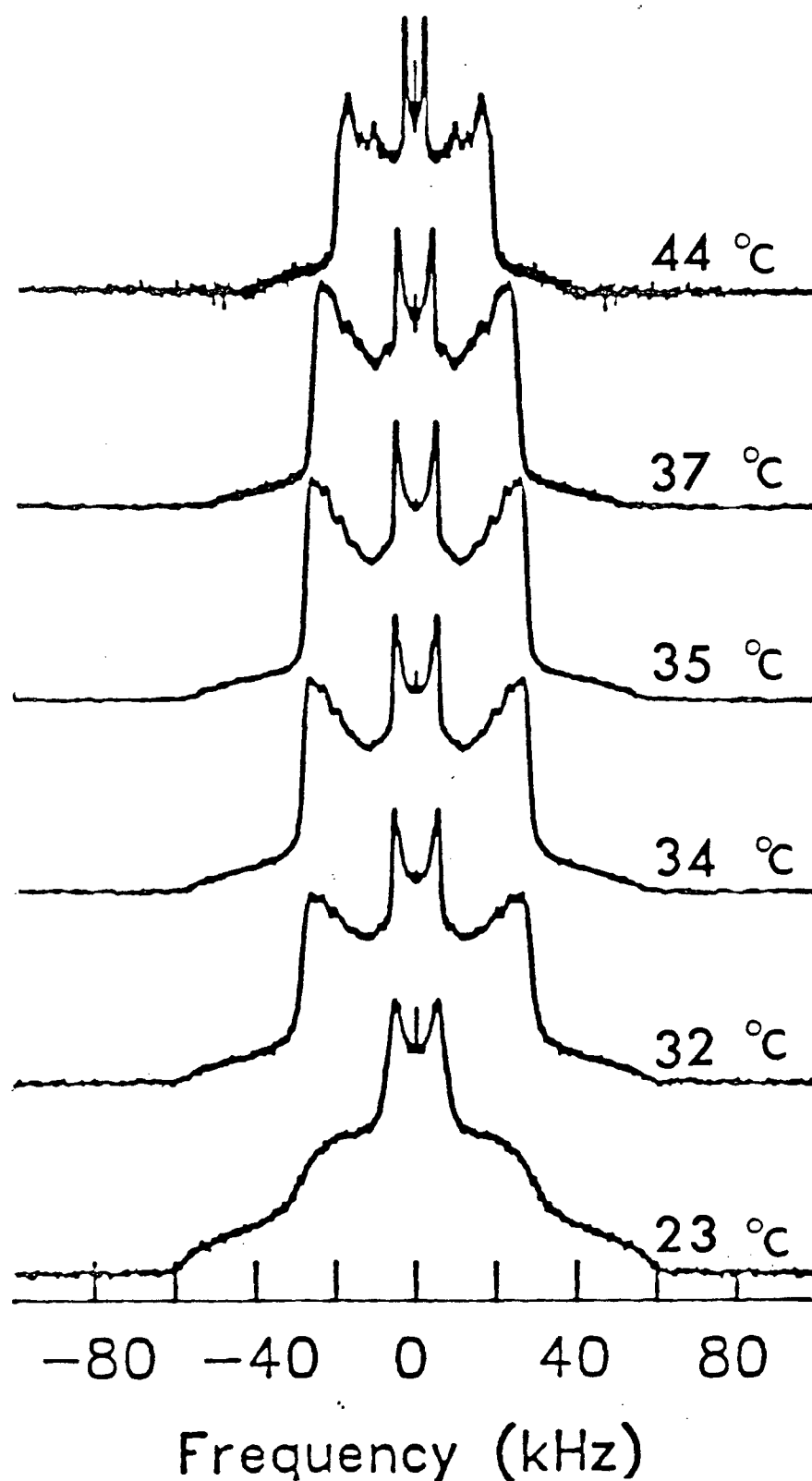
Figure 13. Comparison of the powder pattern spectra for 15% cholesterol/DPPC-d31 measured over a range of temperatures.
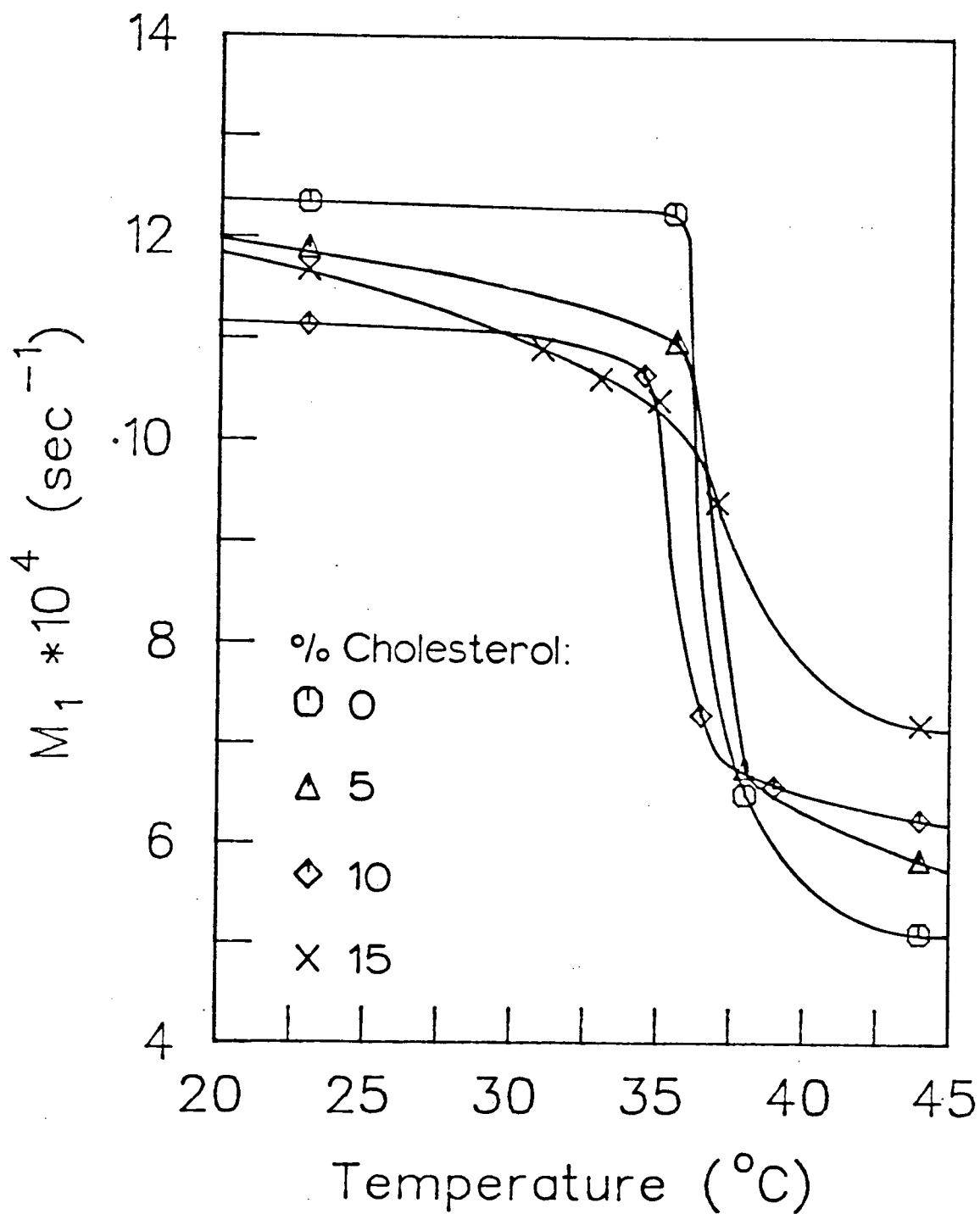
Figure 14. Variation of the first moment, M_1, with temperature for 0, 5, 10 and 15 percent cholesterol concentrations.

specific peaks are influenced by the incorporation of the two sterols. Unfortunately, peak assignments could only be made for the pure DPPC and 5% cholesterol systems at 44°C and the splittings for these resolved peaks are compared in table 1.

Direct comparison can be made between the spectra of pure DPPC-d31, 5% lanosterol and 5% cholesterol at 44°C shown in figure 15. The quadrupole splittings for the two 5% sterol systems differ by only a small amount. However 7 peaks are clearly resolved for the 5% cholesterol spectra while only 5 are resolved for the corresponding lanosterol spectra. The depaked version of figure 15 is presented in figure 16.

Relaxation times have been presented during discussions of the spectral linewidths. T_{2e} is sensitive to slow motions and thus further comparisons can be made between the influences of these two sterol on motions. The relaxation time T_{2e} for the different samples at 44°C can be found in table 2. It is interesting that both sterols result in an increase in T_{2e}, and the increase is greater for cholesterol than for lanosterol. The factor of two increase in T_{2e} with the addition of 5% and 10% cholesterol has also been observed with the addition of certain alcohols [36] .

Figure 15. Comparison of the powder pattern spectra of a) the pure DPPC-d31 sample, b) the 5% lansosterol sample and c) the 5% cholesterol sample all measured at 44°C.
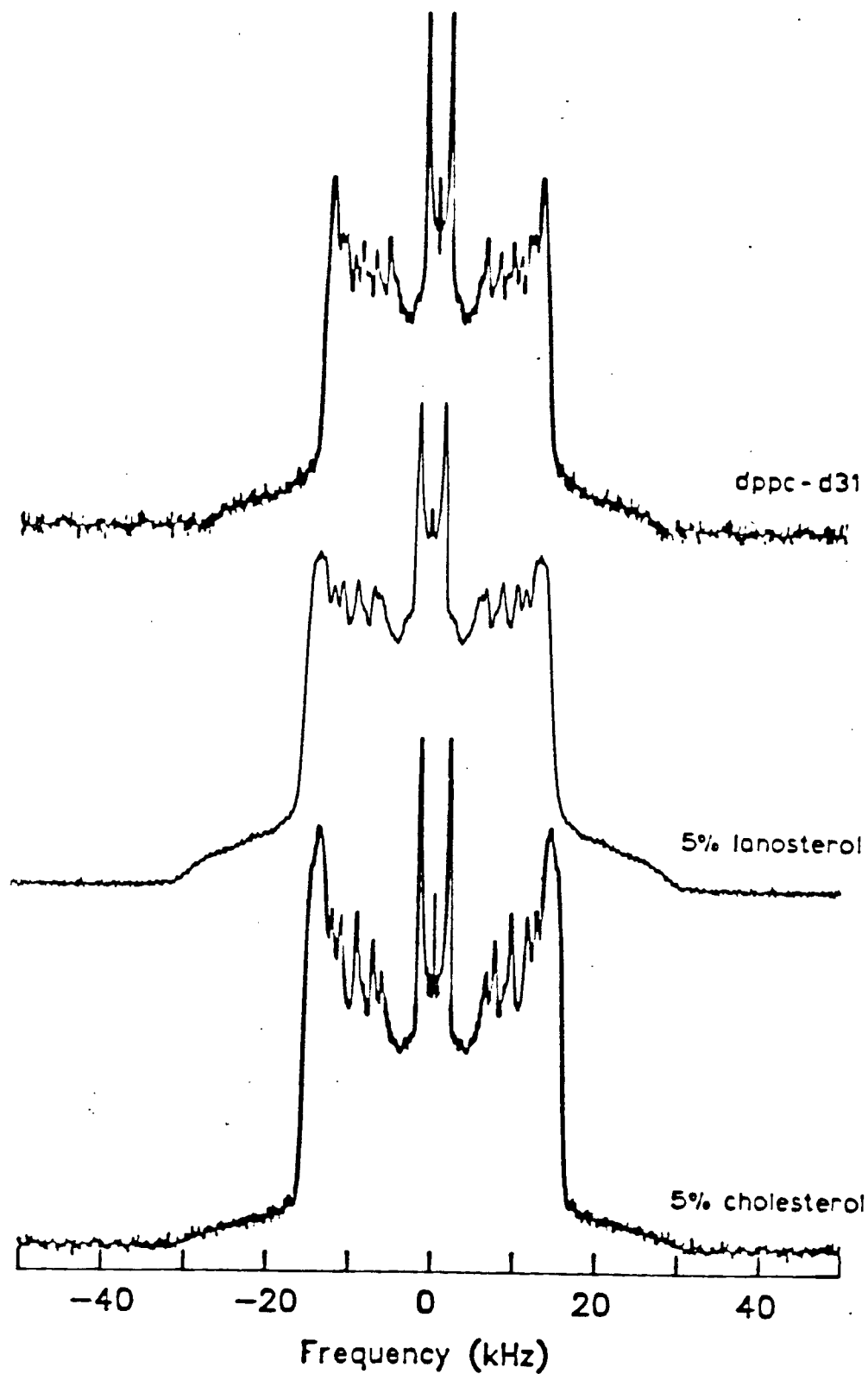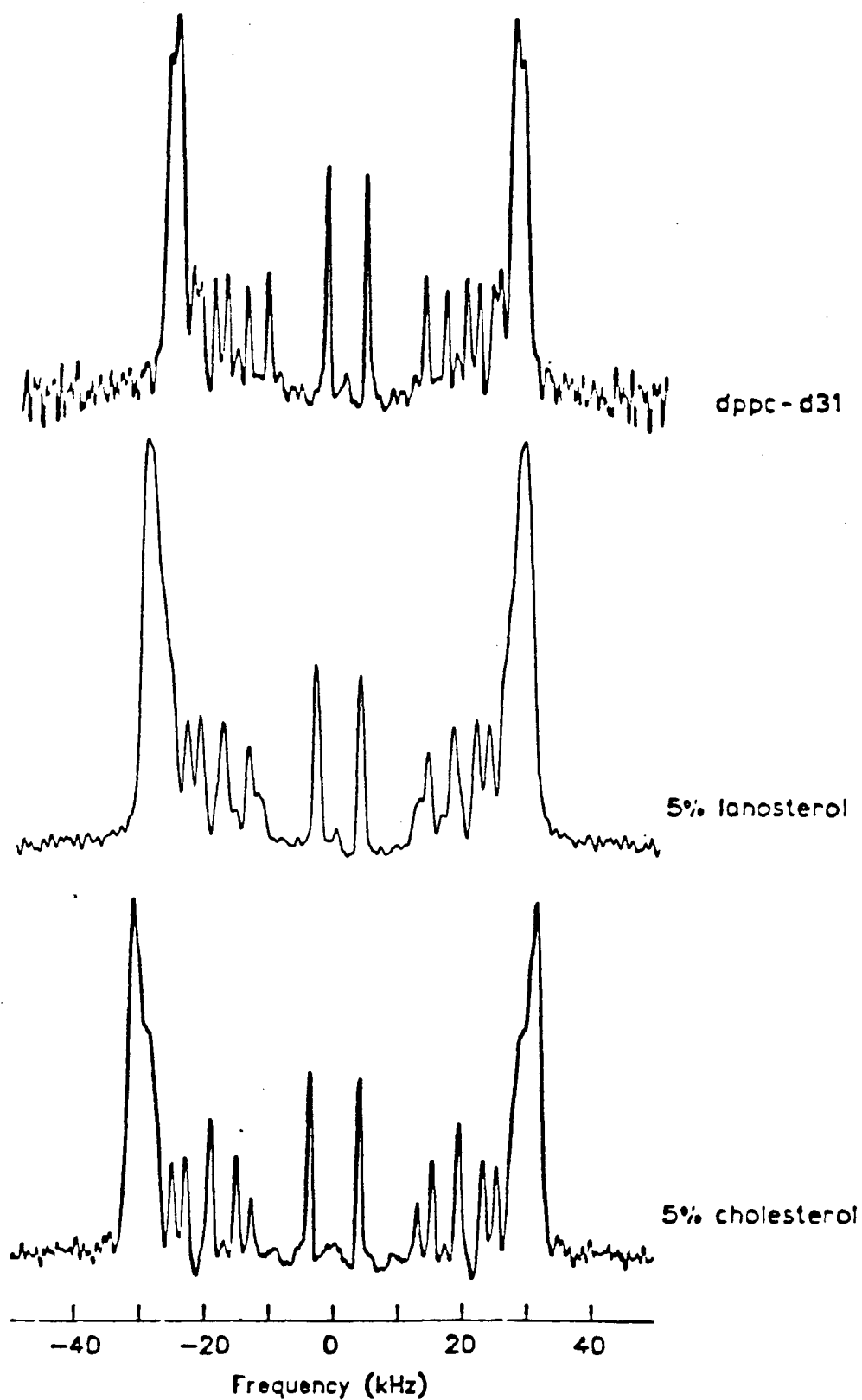
Figure 16. Comparison of the depaked spectra of a) the pure DPPC-d31 sample, b) the 5% lansosterol sample and c) the 5% cholesterol sample all measured at 44°C.

E. DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) was performed on both cholesterol-DPPC and lanosterol-DPPC systems. The transition temperature found for DPPC-d31 using this method was 39°C which is midway between that of pure DPPC (41°C) and DPPC-d62 (37°C [30,40]. Since the calorimetric behaviour of DPPC-d31 was found to be qualitatively the same as that of DPPC, DPPC/sterol systems were compared.

The DSC traces for 0,5,15,20 and 25% lanosterol:DPPC are shown in figure 17. Very similar behaviour is observed for cholesterol (figure 18) and lanosterol. With the addition of 5% cholesterol, the transition temperature is reduced by 1.4°C, whereas the reduction is 1.25°C for the lanosterol transition temperature. Also at this concentration the height of the transition decreases while the width increases. No subtransition was noticeable when lanosterol was introduced.

With the addition of increasing concentrations of lanosterol, the halfwidths increased while the heights further decreased. A constant amount of DPPC was used in each sample and the same scale was used throughout, thus these traces can be compared directly.

The enthalpy of transtition is proportional to the area under the DSC curves. It was found that with increasing concentrations of lanosterol, the enthalpies were reduced. The variation of the enthalpy of transition with lanosterol concentration can be seen in figure 19. A linear decrease in
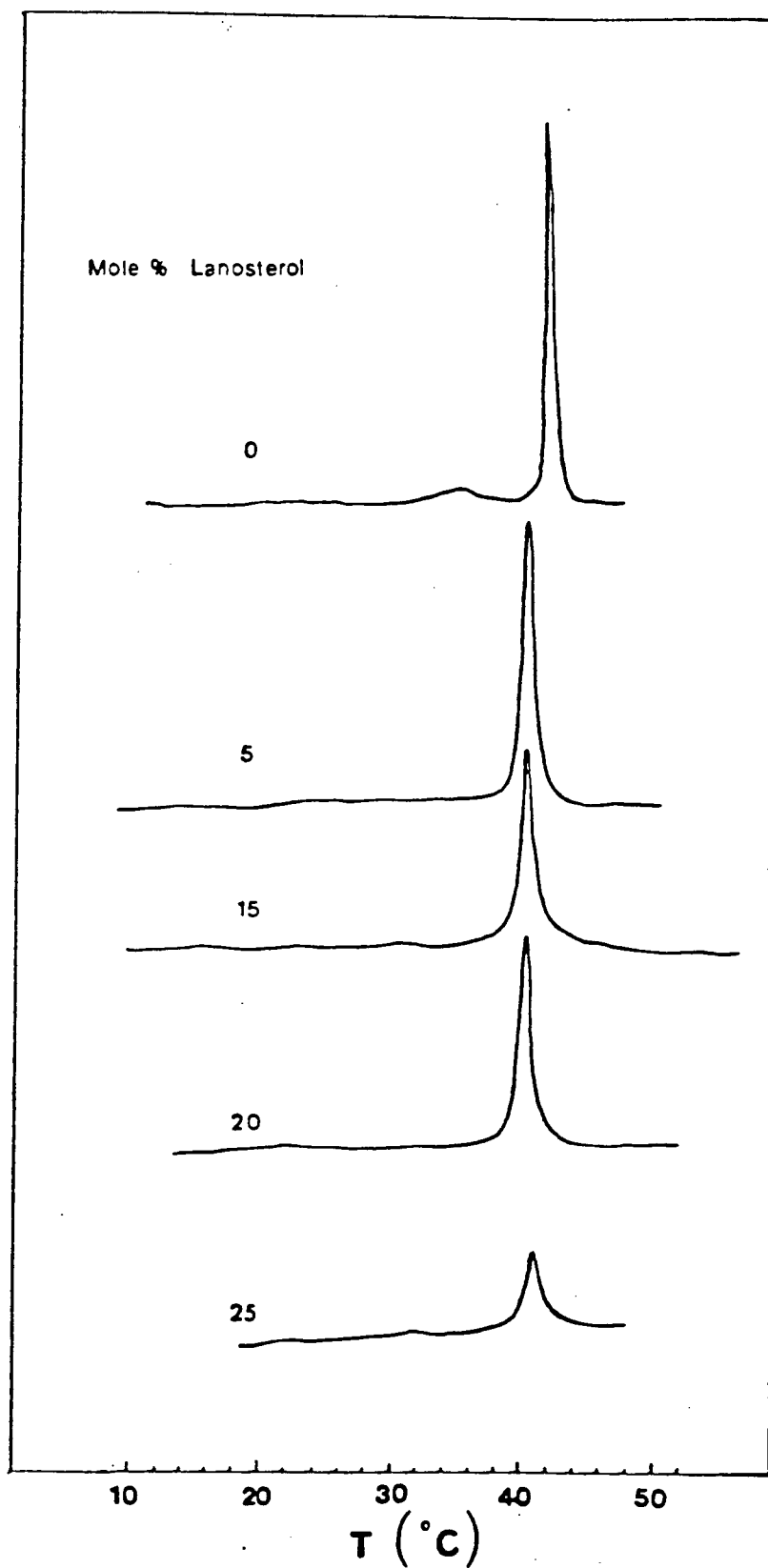
Figure 17. DSC traces of lanosterol/DPPC systems measured at a scan rate of 15°/hr.
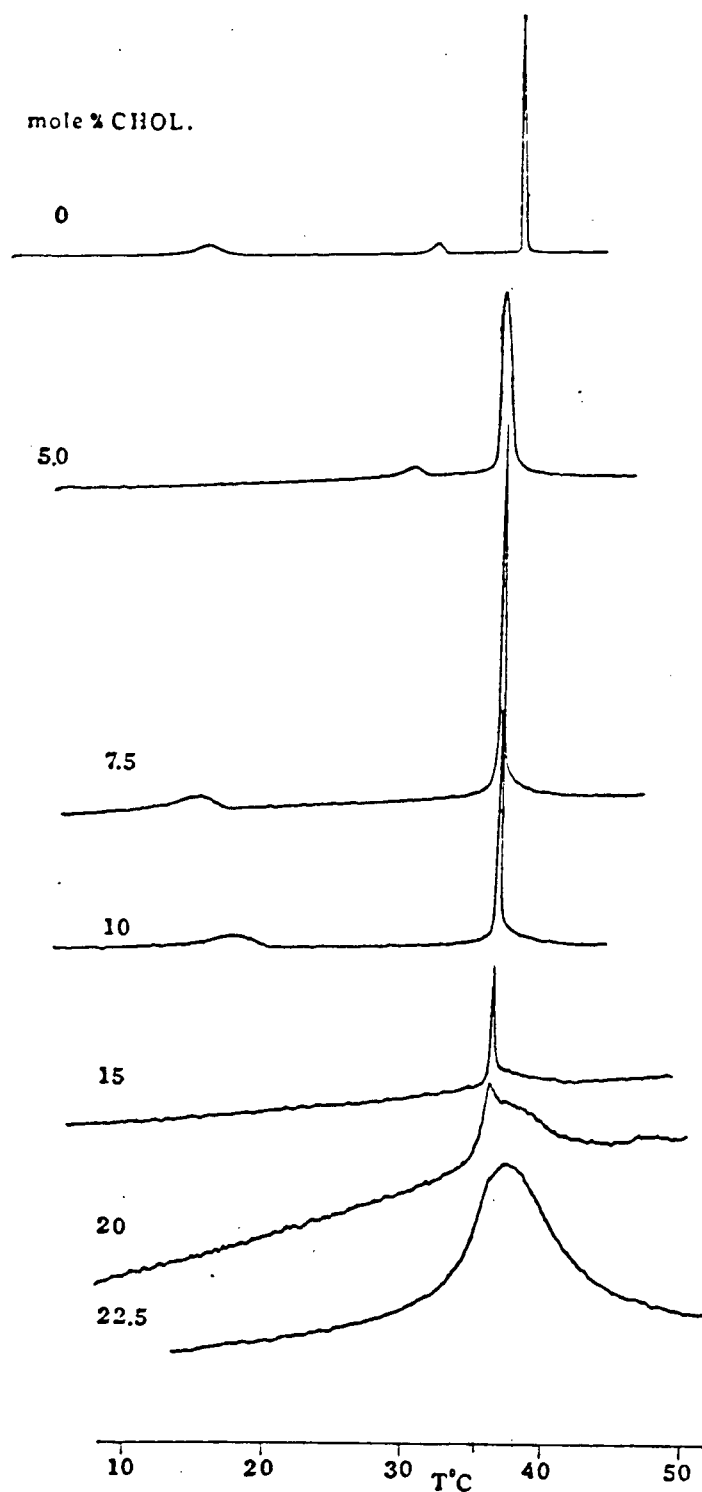
Figure 18. DSC traces of cholesterol/DPPC systems measured at a scan rate of 15°/hr, [6].
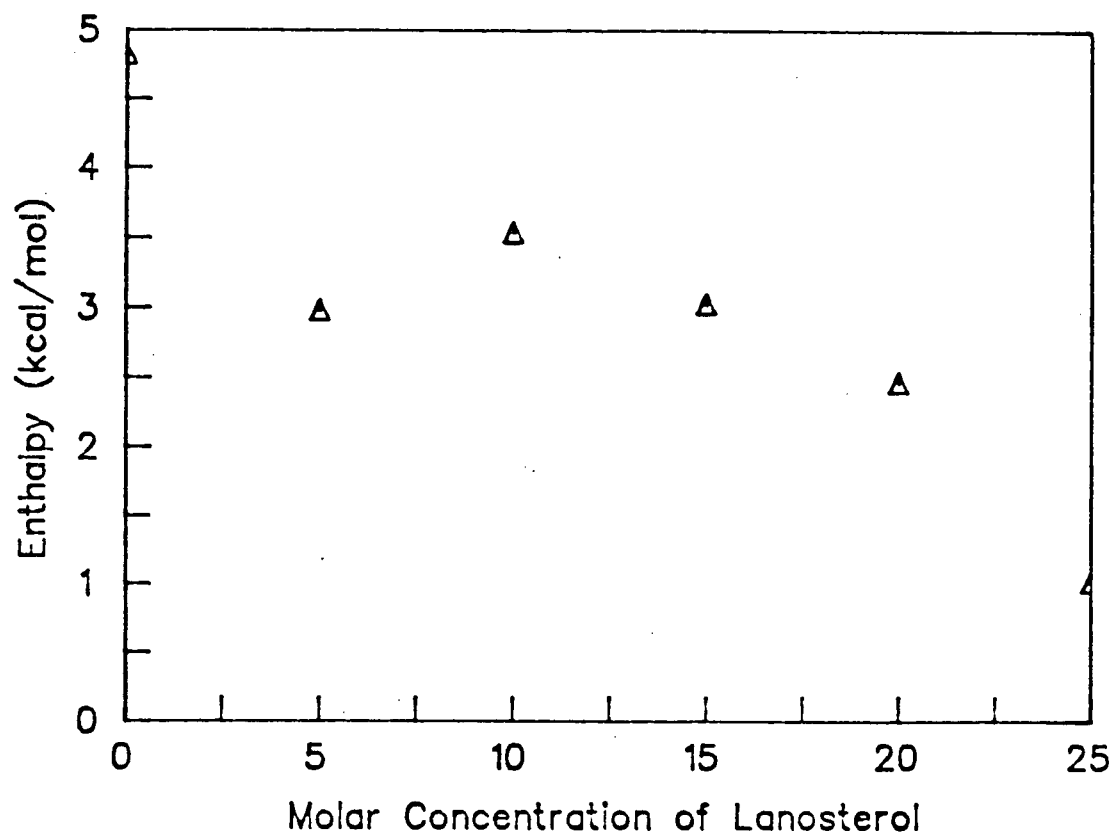
Figure 19. Variation of the enthalpies of transition with sterol concentration.

enthalpy with cholesterol concentration has been observed previously [4,25]. Due to the presence of a broad component the enthalpy measurements contain a large uncertainty. Further, a distortion in the slope of the curves results from the finite scan rate.

V. DISCUSSION

A comparison between lanosterol and cholesterol will be made in terms of the physical properties determined by ^{2}H-NMR and DSC. In addition, the decay time of the quadrupole echo, T_{2e} , will also be discussed in relation to the effect of cholesterol on slow motions.

A. OVERVIEW

With the introduction of oxygen into the atmosphere, the development of membranes involved the incorporation of cholesterol. Lanosterol was present only as a transient step on the evolutionary pathway to cholesterol [1]. The implication of this is that cholesterol bestows superior physical properties on the membranes.

It was hypothesized that if the superiority of cholesterol resulted from the smoothness of the alpha-face, any "bump" on this face (in particular the 14 a methyl group of lanosterol) would seriously inhibit the functioning of the sterol. It was hoped that if this were the case it would be reflected by changes in the NMR spectra.

Carbon positions 9 to 12 are in the region that is likely to be perturbed by the 14-a methyl groups. It is unfortunate that positions 9 and 10 were not resolvable from the plateau region. However, changes in the splittings of the plateau region induced by lanosterol were comparable to the changes observed for cholesterol. In addition the splittings of the peaks that do remain resolved increase

with the incorporation of higher concentrations of both sterols.

A study was performed with dioleoyl phosphatidylcholine (DOPC) specifically labelled at positions 9 and 10 [41]. It was observed that lanosterol and cholesterol had different effects on the chain ordering of DOPC molecules. The incorporation of cholesterol was found to increase the order parameters for methylene deuterons on C_9 but had no effect on the order at position C_{10}. By contrast, lanosterol did not effect the splittings at either position. Some caution is needed in comparing these results with those presented in this work since the splittings given in reference [41] were taken from spectra that were poorly resolved. It should also be mentioned that other studies [6] show that the incorporation of cholesterol increases the quadrupole splittings at all positions down the chain.

A second study using fatty acid spin labels in egg-PC systems also found that lanosterol had less effect on the ordering of methylenes close to the hydrophilic heads than cholesterol and had no effect at positions C_{12} and C_{16} [20].

The systems used in these studies [20,42] contained unsaturated PC lipids which exhibit a marked condensation effect. Medium length fatty chain lecithins such as DPPC do not show such a significant reduction in the cross sectional area with the incorporation of cholesterol [42,43]. Below the transition temperature of the pure lipid, the introduction of cholesterol (<10 mole %) into DPPC membranes

removes the tilt and increases the mobility and amplitude of the motions of the fatty acid chains [44]. Above T_m, by contrast, the system is more ordered than the pure phospholipid system.

It has been proposed that the condensation effect arises from Van der Waals interactions because of the dependence on chain length. Given that DPPC monolayers do not exhibit a marked condensation effect it is possible that the behaviour observed when cholesterol is introduced into the DPPC liposomes is due to the rigidity of the cholestane rings which are oriented perpendicular to the bilayers and restrict the angles the chains can make. Below T_m the system is already ordered and the introduction of cholesterol disrupts the interactions between the acyl chains. It is therefore not surprising that lanosterol exhibited similar behaviour.

B. MOMENTS

As a result of the decreased resolution of the lanosterol/DPPC spectra, it was not possible to compare the splittings of many individual peaks. However a quantitative comparison could be made using the first moments. M_1 is especially useful when discussing the gel and mixed phase regions in which only the methyl peaks are resolved.

Lanosterol and cholesterol systems have very similar moment profiles between 20°C and 45°C at the concentrations examined. Above the phase transition, M_1 increases with

sterol concentration for both systems. This can be interpreted as the rigidifying of the acyl chains.

One may expect lanosterol to be slightly less effective in rigidifying the chains because of the perturbing effects of the extra methyl groups. The observations, which do not agree with this expectation, indicate that the rigidity of the cholestane ring of each of the two molecules is more important than the small protrusion.

Below the phase transition temperature the reverse situation occurs. Both sterols appear to disorder the membranes.

In the two phase region, the fraction of the membrane in the gel phase can be calculated from;

$$M_1 = fM_1{}^G + (1-f)M_1{}^L \qquad\qquad 5.1$$

where f is the fraction of lipids in the gel phase and $M_1{}^G$ and $M_1{}^L$ are the first moments of the gel and liquid crystalline components of the spectrum respectively [45,46] determined at temperatures well away from the transition temperature. This interpretation of M_1 as a linear combination of phases is only valid if the exchange rate between the phases is slow compared to the spectral width.

The fractional composition of the mixed regions can be seen in figure 20. The mixed phase region of the pure DPPC extends over less than 3 degrees whereas with the addition of 5% sterol, the mixed region extends over 10 degrees.
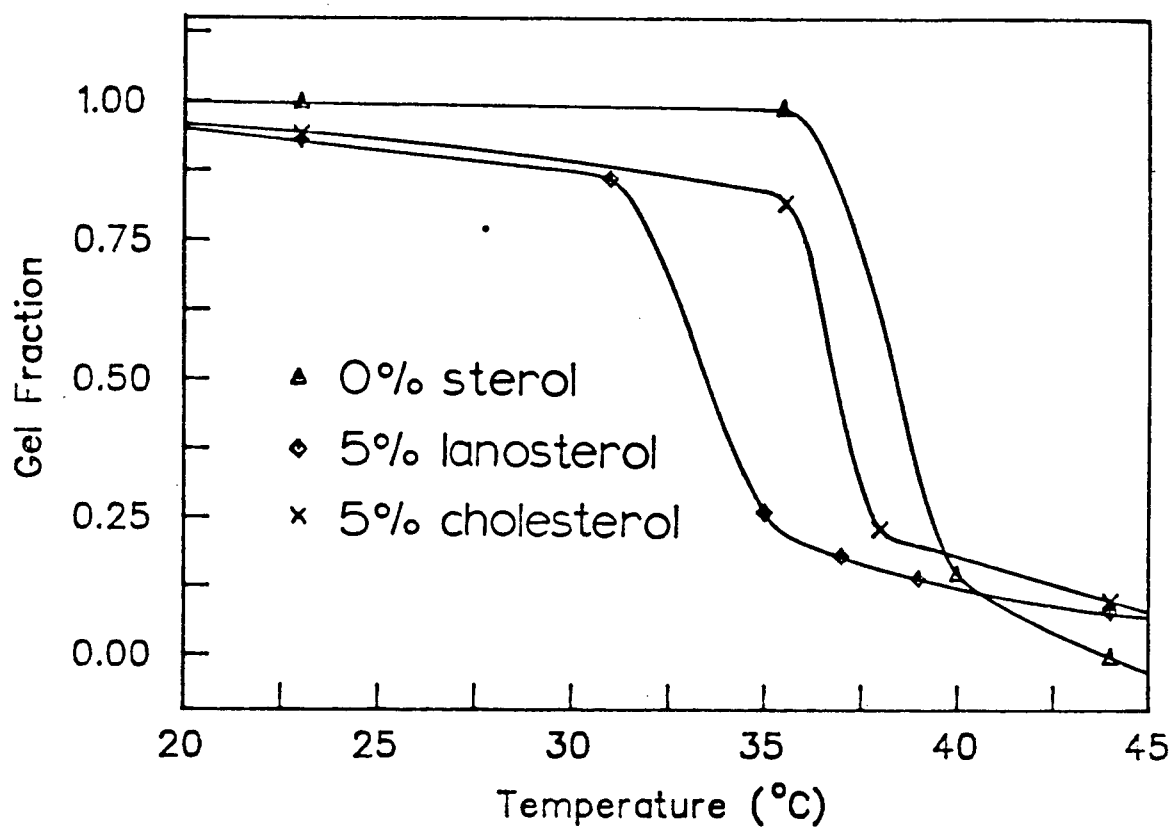
Figure 20. Fraction of DPPC-d31 in the gel phase for the 0% and 5% sterol concentrations as a function of temperature.

The phase diagram for cholesterol (figure 3) shows that at 44°C 5% cholesterol/DPPC membranes are in the liquid crystalline phase. However at the same temperature the 15% cholesterol/DPPC sample is in a mixed L + β phase [6]. This has been described as an average of two phases, liquid crystalline and a second phase which is thought to have a higher concentration of cholesterol than the G and L phases. In this region it was found that the moments (and therefore the spectra) could not be treated as a linear conbination of the two endpoint phases. The reason given for this is that there is fast exchange between the L and β phases. Evidence for the β phase is found in the DSC traces which indicate that at concentrations above 10% , there exists an underlying broad component for both lanosterol and cholesterol/DPPC systems.

At small sterol concentrations, there is a reduction in the transition temperature which is consistent with the proposal that there is a small mixed phase region near the transition temperature of the pure lipid [6] (figure 3).

One reason for studying the phase behaviour is to get some insight into the interactions between the components of the system. Since the phase behaviour of lanosterol at the two concentrations measured is very similar to cholesterol, it is necessary to examine the interactions from another angle.

C. <u>RELAXATION BEHAVIOUR</u>

The variation of the decay of the quadrupole echo determined
for all the samples was exponential for short values of τ.
In the gel phase, T_{2e} was of the order of $100 \mu\text{sec}$. Above T_m
there was a substantial increase in T_{2e}. T_{2e} further
increased with the addition of cholesterol (to 800 μsec) and
to a smaller extent with lanosterol (700 μsec).

It has already been mentioned that T_{2e} is sensitive to
slow motions. The dominant T_{2e} relaxation process of
phospholipid membranes is thought to be associated with slow
motions of the chains which modulate the quadrupole
interactions [30,40]. The addition of sterols into the
membranes may in some way affect the slow motions. A more
definitive interpretation of these results could be made if
a relaxation study were performed which looked at the
relaxation of individual peaks to determine whether or not
the slow motions involve collective motions of the entire
chain.

The significance of the difference in relaxation times
is not known. If it is postulated that the protruding methyl
group is important in modulating the relaxation behaviour,
then this "bump" may be interfering with the motions of the
phospholipids as they pass the alpha face. Since the T_{2e} of
cholesterol is longer, this interpretation implies that the
smooth alpha is important for these motions.

D. RESOLUTION OF THE SPECTRAL LINESHAPES

The difference in T_{2e} values of the 5% lanosterol and cholesterol could account for a small amount of homogeneous broadening of the spectral lines of the lanosterol sample. The broadening of these lines is too great however to be entirely due to relaxation effects, i.e. some inhomogeneous broadening is exhibited. The difference in resolution may be due to the small amount of dihydrolanosterol present in the lanosterol samples. This lipid is present in cells that contain lanosterol.

VI. CONCLUSION

Preliminary studies with systems containing 5%, 10% and 15% sterol/DPPC-d31 indicate that the phase behaviour of lanosterol is consistent with the phase behaviour observed for cholesterol at comparable concentrations and temperatures (figure 3). The phases were assigned using ^{2}H-NMR spectra, their corresponding first moments and from DSC traces.

The width of the transition between the phases at each concentration could be determined from the first moments. With the addition of both lanosterol and cholesterol at a concentration of 5%, the transition temperature was reduced by 1 degree and the width of the transition increased to 4 degrees. This broadening continued at the higher concentration of both sterols. Other authors [6] interpreted the reduction in T_m as the existence of a small mixed phase region near the temperature transition of the pure lipid. If this is the case, both cholesterol and lanosterol exhibit this phase.

The broad component in the DSC traces at sterol concentrations above 10% has been interpreted by the same authors [6] as a "β" phase. Both sterols appear to effect this phase.

Further, the first moment was used to determine the fraction of phospholipid in the gel phase at the 5% concentration. This result indicated that at the concentrations examined, lanosterol and cholesterol have the

60

same composition of gel phase lipids.

Above the phase transition of the pure phospolipid, samples containing either lanosterol or cholesterol are more ordered than the pure phospholipid systems at the same temperature. Therefore the presence of either sterol restricts the formation of gauche bonds, "condensing" the phospholipids. This disagrees with statements by Dahl *et al* [20]

The protrusion from the alpha face of lanosterol does not impair the ability of the sterol to order phospholipids nor does it alter the phase behaviour. These observations may be attributable to the rigidness of the ring structure which is possessed by both sterols .

The T_{2e} relaxation times are slightly different for lanosterol and cholesterol. At low concentrations of cholesterol T_{2e} values increased by a factor of two over pure DPPC. This suggests cholesterol is speeding up the slowest motion of the phospholipid. The smoothness of the a-face of cholesterol may allow phospholipids to slide by, unimpeded by any protrusion. If this were the case the a-methyl on lanosterol would hinder the movement of the phospholipids.

To determine more clearly the nature of the slow motions modulating T_{2e} a more extensive relaxation study should be performed, including the observation of how the individual spectral lines relax.

The difference in resolution of the two sterols could not be explained completely in terms of changes in T_{2e}. One possible reason for the decreased resolution may be that the small amounts of dihydrolanosterol in the samples contribute to the inhomogeneous line broadening.

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