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Thermal analysis of phase transition behaviour in liposomes

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Abstract

Liposomes are small vesicles having one or more concentric phospholipid bilayers, interspersed with aqueous phase. Thermal analysis, particularly differential scanning calorimetry (DSC) has been used extensively to study the behaviour of hydrated phospholipids within bilayers. On heating, and dependent on the state of hydration, phospholipids undergo a series of phase transitions. This paper reviews investigations of the phase transition behaviour of single component phospholipid systems, bilayers having mixtures of phospholipids and bilayers containing phospholipid–cholesterol mixtures. Liposomes are being extensively studied for use as colloidal drug delivery systems. Inclusion of therapeutic agents into liposomal systems may have pronounced effects on the phospholipid phase transition behaviour and consequently bilayer fluidity. These effects are determined by the location of the therapeutic agent within the bilayer. The interactions of electrolytes and steroids with phospholipids are discussed. The use of DSC studies of drug–liposome interactions as models of drug–cell membrane interactions is also discussed.

Keywords: Drug; DSC; Liposome; Model; Phase transition

1. Introduction

Liposomes are phospholipid vesicles, having diameters of between 20 nm and 20 μm. The amphiphilic phospholipid molecules are arranged in one or more concen-

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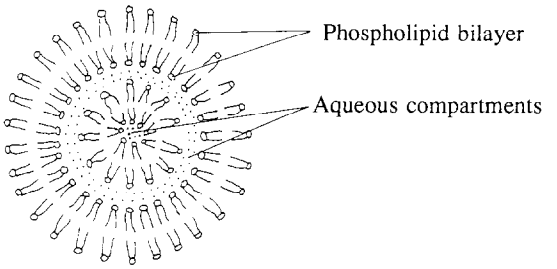

Fig. 1. A multilayer liposome: the polar head groups of the phospholipid molecules are oriented towards the water interface. (Reproduced with permission from Ref. [1].)

tric bilayers separated by aqueous channels, surrounding an aqueous core (Fig. 1). Following their description by Bangham et al. [2], they attracted considerable interest as model membranes due to their structural similarity to cell membranes. More recently, liposomes have been extensively studied as potential drug carrier systems to modify the pharmacokinetic behaviour of therapeutic molecules.

A number of phospholipids may be used to manufacture liposomes, which may be broadly classified into natural phospholipids (e.g. phosphatidylcholine (PC), phosphatidylserine and phosphatidylglycerol from egg yolk or soya beans and sphingomyelin) and synthetic phospholipids (e.g. distearoylphosphatidylcholine (DSPC) dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC) and dilaurylphosphatidylcholine (DLPC)).

The traditional method of liposome production requires addition of aqueous medium to a phospholipid film at an appropriate temperature. Agitation results in the generation of multilamellar vesicles (MLVs), which have more than one bilayer surrounding the aqueous core (Fig. 1). These liposomes are comparatively large, having diameters between 0.5 μm and 20 μm. Other liposome types have been described, including small unilamellar vesicles (SUVs), which contain only one phospholipid bilayer and have diameters between 20 and 100 nm. SUVs may be produced by sonicating MLVs [3], by injecting an ethanolic solution of phospholipid into an aqueous phase [4], or by dialysing detergent-containing phospholipid solutions [5]. Large unilamellar vesicles (LUVs) range in size between 100 nm and 1 μm and may also be produced by the solvent-injection method using suitable solvents [6]. Szoka and Papahadjopoulos [7] have produced liposomes known as reverse-phase evaporation vesicles (REVs). An aqueous phase is emulsified in the presence of phospholipids dissolved in organic solvents. Evaporation of the organic solvent under vacuum produces LUVs and large liposomes with two or three bilayers. LUVs have a higher efficiency of aqueous drug entrapment than MLVs or SUVs.

2. Phase transitions of single component phospholipid systems

On heating, phospholipids do not undergo a simple melting process, passing from solid to liquid state, but depending on the amount of water present, exist in one or more intermediate liquid-crystalline or mesomorphic forms.

The most common phospholipids employed in the production of liposomes are the phosphatidylcholines. These have been extensively investigated by thermal means. Differential scanning calorimetry (DSC) is the technique which has been most widely applied to liposomal systems. The parameters derived from DSC analysis include the onset temperature of the transition (T_0), and the temperature at the peak (T_m), frequency denoted T_c when referring to the gel to liquid-crystalline phase transition. The width of the transition at half peak height ($\Delta T_{1/2}$ or HHW) and the enthalpy of the process (ΔH) are also derived.

Differential thermal analysis (DTA) has indicated that 1,2-diacyl-L-phosphatidylcholines are isolated from organic solvents in the monohydrate form [8], and that this water is lost on heating samples in open DTA pans. This is associated with a large endothermic transition, many degrees below the melting point. Addition of water to DSPC lowers the temperature at which the main gel to liquid-crystalline phase transition occurs, a limiting value being reached at approximately 20% w/w water content [9]. An endothermic peak corresponding to the melting of ice is not observed until the water reaches 25% w/w (Fig. 2). A plot of heat absorbed in ice melting against concentration extrapolates to zero at 20% w/w water content. At this concentration, equivalent to approximately 10 molecules of water to one molecule of phospholipid, water is bound to phospholipid molecules and does not freeze. The main gel to liquid-crystalline phase transition of DSPC becomes sharper and more defined on addition of water compared to dry phosphatidylcholine and corresponds to liposome formation (Fig. 2).

High sensitivity DSC analysis of liposomal phosphatidylcholine bilayers, indicates a number of endothermic transition peaks. Following prolonged incubation at low temperatures, fully hydrated, long chain phosphatidylcholines, such as DPPC exist in an ordered, condensed crystalline subgel (L_c) state, in which the hydrocarbon chains are in the fully extended, all trans conformation and the polar head groups are relatively immobile [10–12]. On heating, L_c state synthetic phospholipids undergo a transition (the subtransition) to the $L_{\beta'}$ state, in which the polar head groups have increased mobility, and there is an increased penetration of water into the interfacial region of the bilayers [11–13]. Subtransitions may be classified into two groups. Type I "solid–solid" transitions between subgel and gel phase are found in saturated phosphatidylcholines with C_{16} to C_{18} chains [14,15] and in dipalmitoylphosphatidylglycerol [16] and are characterised by a very small change in rotameric disordering. Type II transitions involve much more rotameric disordering and more melting of the subgel phase into the liquid-crystalline phase. Saturated phosphatidylethanolamines exhibit such transitions [14,17]. Equimolar mixtures of phosphatidylcholines have been reported not to exhibit subtransitions, although the individual constituents do [15]. Head group interactions also seem important in such transitions, because addition of small amounts of the opposite stereoisomer (5% D-DPPC) or cholesterol prevents the subtransition [18]. A sub-subtransition has been described for DPPC with a T_m at 6.8°C [18]. A prolonged low temperature incubation is not required to observe this transition, and the sub-subgel phase is seen as a precursor to the subgel phase, forming immediately on cooling the gel phase [18].

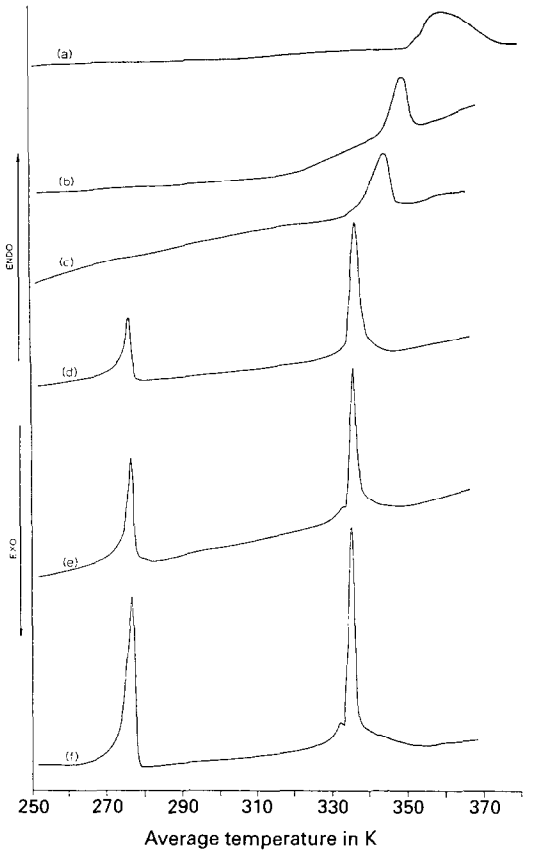

Fig. 2. DSC curves for DSPC as a function of hydration: curve (a), anhydrous; curve (b) 10% w/w water; curve (c), 20% w/w water; curve (d), 25% w/w water; curve (e), 30% w/w water; curve (f), 40% w/w water. (Reproduced with permission from Ref. [9].)

Heating of $L_{\beta'}$ state phospholipids results in conversion (the pretransition) to the gel ($P_{\beta'}$) state. The pretransition has been attributed to rotation of the polar head groups of the phospholipid molecules or due to a co-operative movement of the rigid hydrocarbon chains prior to melting [19]. However, the pretransition of the synthetic phospholipids DMPC and DPPC has been shown to be due to structural changes in the lamellar lattice [20]. At temperatures below the pretransition a one dimensional lamellar lattice exists, with the hydrocarbon chains of the phospholipids fully extended and tilted with respect to the plane of the bilayer. The pretransition is associated with a structural transformation from a one dimensional lamellar to a two dimensional monoclinic lattice consisting of lipid lamellae distorted by periodic "ripples".

Heating $P_{\beta'}$ state phospholipids induces the co-operative "melting" of the hydrocarbon chains (the main gel to liquid-crystalline phase transition) to produce the L_{α}

Table 1
Gel to liquid-crystalline phase transition temperatures for phosphatidylcholine bilayers (source, Ref. [9])

Lipid	Transition temperature/°C
DLPC (C_{12})	0
DMPC (C_{14})	23
DPPC (C_{16})	41
DSPC (C_{18})	58
DBPC (C_{22})	75
Egg PC	−5 to −15
Soya PC	−20 to −30

state. The nature of the main phospholipid bilayer transition has been comprehensively described by Nagle [21]. The transition is related to the melting of the hydrocarbon chains. Below the T_c the hydrocarbon chains are parallel and the C–C bonds are in the trans configuration. At the T_c there is disordering of the chains due to trans to gauche conformational changes of the C–C bonds.

Phospholipid molecules are packed into bilayers, such that the mobility of the hydrocarbon chains is restricted; with one end of the hydrocarbon chain attached to a polar head group, held at the water interface. This anisotropic restraint reduces the amount of disordering occurring at the phase transition compared to the isotropic melting of alkanes. Chain rotation is forced to be a co-operative event, in a direction perpendicular to the bilayer surface. The gel to liquid-crystalline phase transition temperature has been measured by DSC for a number of synthetic and naturally occurring phospholipids (Table 1). Naturally occurring phospholipids are usually mixtures of components having different length hydrocarbon chains. Such mixtures would usually be expected to produce broad ill defined transitions (see below). Egg phosphatidylcholine comprises predominantly C_{16} and C_{18} chain phospholipids [22]; consequently a relatively broad but fairly well defined transition can be measured for this lipid. The T_c generally depends on the nature of the polar head group and the length and degree of unsaturation of the hydrocarbon chains. For phospholipids with the same head group and degree of hydration, increasing saturation in the hydrocarbon chains increases the T_c [23], with trans-unsaturated chains having a higher T_c than those which are cis-unsaturated [24]. Phospholipids with longer hydrocarbon chains have a higher T_c than those with shorter ones [8]. Menger et al. [25] have shown that adding a methyl branch to a phospholipid hydrocarbon chain has a much more pronounced effect mid-chain than at either end. This they suggest is due to carbons in that part of the chain nearest the head group being relatively immobilised, with chains remaining linear even if a methyl group is added, whilst carbons at the terminal end of chains are already mobile. Addition of a methyl group at the centre of a chain is most likely to induce a "kink", which is essential for the main transition. Phosphatidylethanolamines exhibit no demonstrable pretransitions and have T_c values higher than the equivalent phosphatidylcholines [23], which is attributed to a stronger head group

Table 2
Thermodynamic data for the gel to liquid-crystalline transition in sonicated and unsonicated DMPC and DPPC liposomes (adapted from Ref. [27])

	DMPC		DPPC	
	Unsonicated	Sonicated	Unsonicated	Sonicated
Transition temperature/°C	23	12	41	32
Transition enthalpy/kcal mol^{-1}	6.6	3.3	8.6	6.0
Transition entropy/cal mol^{-1} $°C^{-1}$	22	11	28	19

1 J is 0.239 cal.

interaction. Phosphatidylethanolamine L_α phases convert to H_{II} phases on heating. In this form, the phospholipid molecules are arranged in long water-cored tubes that stack on a two dimensional lattice [26].

Sonication of multilamellar liposomes above the phospholipid T_c to produce SUVs may disrupt the regular packing of bilayers and impose constraints on the phospholipid molecules due to the small radius of curvature of the vesicles. Melchior and Stein [27] have shown that for SUVs the main transition endotherm is lowered and increased in width with a decreased enthalpy and entropy (Table 2). Broadening of transitions following sonication has also been demonstrated using fluorescent probes [28] and dilatometry [29].

3. Phase transitions of multi-component phospholipid systems and phospholipids with hydrocarbon chains of different lengths

Biological membranes and many liposomal formulations contain a mixture of phospholipids, and consequently it is important to understand the phase behaviour of mixed systems. DSC studies have indicated that mixtures of phospholipids, containing different hydrocarbon chains melt over a broader temperature range than pure lipids [30]. The T_c and the shape of the transition are dependent on the lipid composition, and asymmetric transitions occur when compositions other than equimolar are used. When the hydrocarbon chains differ by only two carbon atoms (e.g. C_{14} and C_{16} or C_{16} and C_{18}) ideal mixing of phases occurs [30,31]. Mixing DMPC and DPPC produces asymmetric transitions intermediate between the two components (Fig. 3) with phase diagrams constructed from initiation and completion transition temperatures indicating complete miscibility in both liquid and solid phases [30]. A difference of four carbon atoms in the hydrocarbon chains (e.g. C_{14} and C_{18}) results in a system significantly moved from ideality, although Mabrey and Sturtevant [30] were not able to detect monotectic behaviour. However, other DSC studies have suggested that regions of gel phase immiscibility occur, possibly with peritectic behaviour [32,33]. A mixture of phospholipids, in which the hydrocarbon chains differ by six carbon atoms (e.g. C_{12} and C_{18}) results in a system so removed from the ideal that monotectic behaviour is observed (Fig. 4). For a given differ-

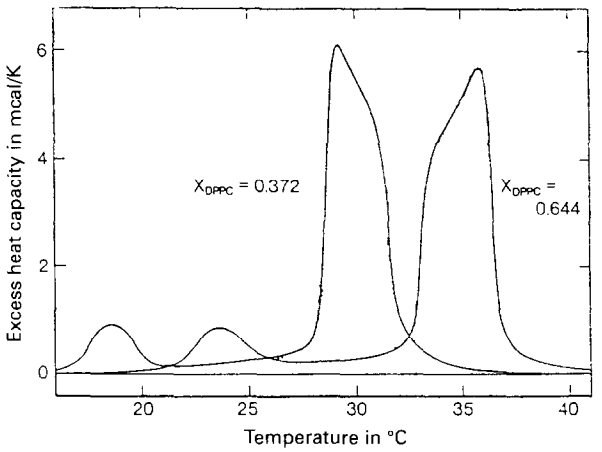

Fig. 3. DSC curves for multilamellar liposomes containing mixtures of DMPC and DPPC. (Reproduced with permission from Ref. [30].) One mjoule is 0.239 mcal.

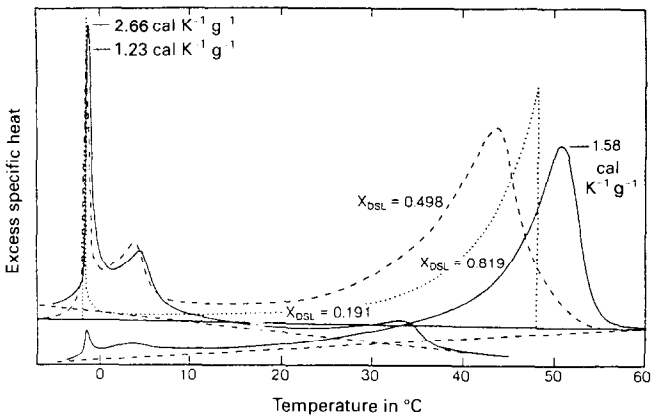

Fig. 4. DSC curves for multilamellar liposomes containing mixtures of DLPC and DSPC (DSL). Solid curve (dashed baseline) corresponds to $X_{DSPC} = 0.191$, dashed curve, (solid baseline) corresponds to $X_{DSPC} = 0.819$, dotted curve corresponds to $X_{DSPC} = 0.498$. (Reproduced with permission from Ref. [30].) One joule is 0.239 cal.

ence in the length of the hydrocarbon chains, substitution of the phosphatidylethanolamine for the phosphatidylcholine in the lower melting component does not increase the tendency towards phase separation [30].

There have been a number of studies of bilayers containing highly asymmetric phosphatidylcholine molecules. Hydrated phosphatidylcholines having one hydrocarbon chain approximately twice as long as the other, for instance C(18):C(10) PC, exhibit a single highly co-operative phase transition over a wide temperature range indicating the existence of a single type of gel phase below the T_c. This gel phase is designated the interdigitated phase, in which the long hydrocarbon chains

from opposing monolayers interpenetrate across the hydrocarbon width of the bilayer, whilst opposing short chains pack "end to end" [34]. The transition behaviour of interdigitated bilayers is more sensitive to inclusion of cholesterol (see below) than non-interdigitated bilayers [35]. Strict structural conditions, which are no longer satisfied in the presence of cholesterol, are necessary to achieve interdigitation; these cause local disordering in the gel phase of C(18):C(10) PC liposomes. Heating and cooling thermograms indicate that T_0 and T_m decrease with increasing cholesterol content [35]. The decrease in temperature is approximately $-0.24^{\circ}C$ per mol% cholesterol, greater than the equivalent symmetrical phosphatidylcholine–cholesterol mixtures.

4. Phospholipid–cholesterol mixtures

Cholesterol is frequently included in liposome formulations to control the rate of release of entrapped hydrophilic materials [36] or to increase their in vivo and in vitro stability [37]. Addition of cholesterol to DPPC bilayers at concentrations greater than 20 mol% results in elimination of the pretransition and a decrease in the main transition temperature (Fig. 5). The DSC endotherm broadens and the

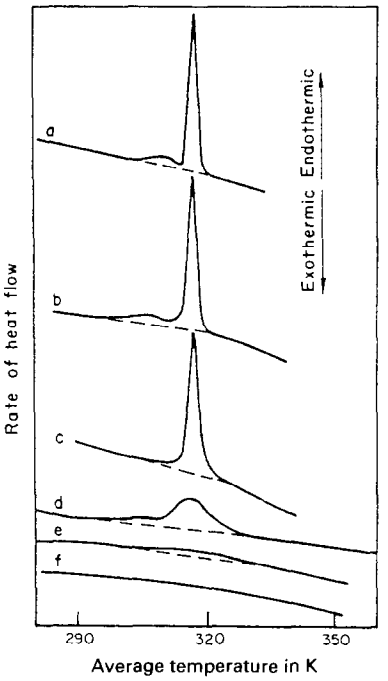

Fig. 5. DSC curves for dispersions of DPPC in water containing cholesterol: curve a, 0 mol%; curve b, 5 mol%; curve c, 12.5 mol%; curve d, 20 mol%; curve e, 32 mol%, curve f, 50 mol%. (Reproduced with permission from Ref. [23].)

area under the curve, i.e. the heat of transition, decreases [23,38]. A distinct transition is not detectable by thermal means at 50 mol% cholesterol [38,39], which is the maximum concentration of cholesterol that can be incorporated before phase separation occurs. Addition of cholesterol to phospholipid bilayers "modulates" their fluidity [23]. Below the T_c, in the presence of cholesterol, the phospholipid chains are more mobile than in the absence of cholesterol as the cholesterol prevents the hydrocarbon chains crystallising into the rigid crystalline gel phase. Above the T_c the rigid sterol molecule restricts the movement of the hydrocarbon chains. Although at high cholesterol concentrations, a transition cannot be detected using DSC, laser Raman studies have indicated that a non-cooperative transition does take place over a very wide temperature range [40].

5. Liposomes as drug delivery systems and model membranes

The amphiphilic nature of liposomes permits their use as drug delivery systems for hydrophilic materials (entrapped in aqueous regions) and hydrophobic materials (located in the hydrocarbon region within bilayers. Amphiphilic drugs partition between these two regions [41]. Phase transition behaviour is important in considering the application of liposomes for drug delivery, since the permeability of bilayers to entrapped hydrophilic materials increases as the bilayer becomes more fluid, an important factor when considering the stability of the dosage form and the rate of drug release in vivo.

Thermal analysis of the interaction between drugs and phospholipid bilayers has been used as a method to investigate the action of drug molecules on biological membranes. For instance, the activity of antibiotics such as chlorothricin and gramicidin A [42] and polymyxin B [43] has been assessed using DSC studies of their interactions with DPPC liposomes. Although the bacterial cell membrane is chemically complex and does not contain DPPC, the interaction between the drugs and the bilayer is similar and therefore DPPC liposomes present a simple, easily characterised model.

It has been proposed that some anesthetics produce anaesthesia by increasing the fluidity of nerve cell membranes, which in turn alters the functioning of sodium channels [44]. Clearly, DSC studies of liposomal models offer the potential to investigate this effect. Increasing the concentration of the local anesthetics benzocaine and dibucaine added to DPPC bilayers, caused a progressive movement of the main transition to lower temperature, whilst the width of the transition remained approximately unchanged [45]. However, caution is necessary when attempting to correlate anaesthetic activity of a compound with its effect on the gel to liquid-crystalline transition in a simple liposomal model, because a number of studies with non-volatile general anaesthetics and steroids have indicated that no direct relationship can be demonstrated between their therapeutic potency and the effect on transition behaviour [46,47].

Jain and Wu [48] used DSC to investigate the effect of more than 90 materials on the phase transition behaviour of DPPC liposomes. A number of compounds were

investigated including fatty acids, alcohols, electrolytes, detergents, organic solvents, ionophores, spin labels and fluorescent membrane probes. The authors proposed that the effect on the phase transition was determined by the physical location of the additive within the bilayer. The packing of the gel phase necessitates that the C–C bonds in the hydrocarbon chains be in the all-trans state, whilst in the liquid-crystalline state, some of these bonds assume the gauche configuration. The mobility of the chains and the probability of the gauche conformation increases along the hydrocarbon chain away from the head group. Up to C_9 there is considerable segmental packing, such that the highly cooperative nature of the main phase transition is regulated by the interaction of the C_1–C_{10} bonds. Consequently the influence of an additive on the transition is influenced by its position in the bilayer, especially if located in the C_1–C_{10} region.

Jain and Wu [48] distinguished at least four different types of modified transition, related to changes in membrane fluidity. These they categorised as follows:

Type A. Additive localised in the C_1–C_8 methylene region (usually long molecules with polar and non-polar regions, e.g. higher alcohols and some detergents).

Type B. Additive localised in the glycerol backbone of the phospholipid molecules (usually relatively large disc shaped, asymmetric, relatively polar molecules).

Type C. Additive localised in the C_9–C_{16} methylene region (small dipolar compounds, e.g. carbon tetrachloride, chloroform).

Type D. Additive interacts with the polar head group (electrolytes).

These different classes of compounds have increasing polarity Type C < Type A < Type B < Type D; i.e. a Type C compound would be localised furthest from the interface and a Type D compound closest to or at the interface. This is qualitatively consistent with their effect on transition behaviour.

Cations are believed to interact electrostatically with the head groups of phospholipid molecules. The most pronounced effect occurs with di- and trivalent cations [48]. The presence of Mg^{2+}, Ca^{2+}, Pr^{3+}, Eu^{3+} and Gd^{3+} ions modify the phase transition profile of DPPC (Fig. 6). Addition of these cations results in a reduction in the original transition peak and the appearance of a new peak at a higher temperature. At very high solute concentrations the parent peak is completely removed. Interaction of multivalent cations will produce significant effects on the packing of phospholipids within the bilayer due to rearrangements of the zwitterionic head group [49]. It is suggested that addition of divalent ions results in a modified phase, which has the same enthalpy of transition and the same size co-operative unit as the unmodified bilayer phase [48]. Monovalent cations do not significantly affect the transition behaviour of DPPC bilayers [48].

A number of workers have studied the interaction between liposomes and steroids, both because liposomal steroid formulations may have applications in the treatment of diseases such as rheumatoid arthritis [50] and because the pharmacological action of steroids is believed at least in part to derive from their interaction with membrane phospholipids [51]. Additionally, steroids and homologous series of their esters offer a good model system for investigating the interaction of relatively

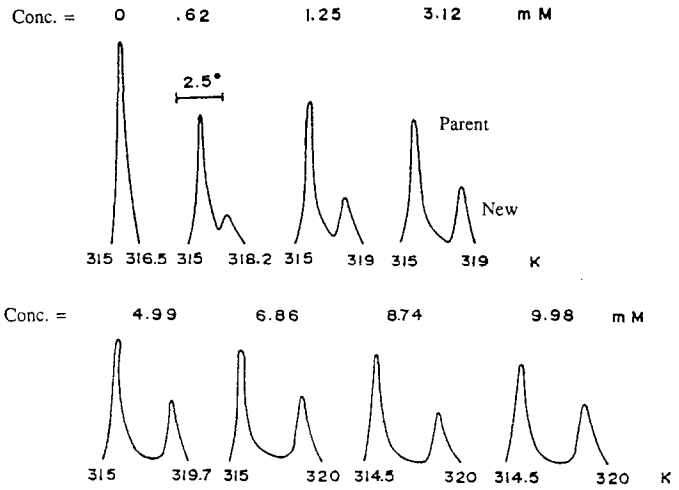

Fig. 6. Modification of the phase transition profile of DPPC by addition of increasing concentrations of gadolinium chloride ($GdCl_3$). The horizontal bar corresponds to 2.5 K. (Reproduced with permission from Ref. [48].)

hydrophobic materials with bilayers. Increasing the concentration of steroids incorporated into liposomes results in a broadening of the transition peak. Fildes and Oliver [52] measured the width of the DSC transition peak at half its height (HHW) to maximise the incorporation of hydrocortisone-21-palmitate in DPPC liposomes. The use of the HHW criteria had previously been advocated as a measure of the interaction between compounds and phospholipid bilayers [48,53]. Inclusion of 3.8 mol% or greater of hydrocortisone palmitate into liposomes abolished the pretransition. The temperature of the main phase transition was considered independent of steroid content, although the HHW increased to a maximum of 9° at 13.2 mol% before decreasing, indicating the maximum level of drug incorporation, i.e. bilayer saturation. This provides one of the few methods available for determining the amount of hydrophobic material incorporated into liposomes. Arrowsmith et al. [54] used DSC to investigate the interaction between DPPC MLVs and a series of cortisone esters. HHW and T_c were used to measure the liposome–steroid interaction. The extent of interaction increased with increasing concentration and increasing chain length. Saturation of the bilayer occurred with 11.25 mol% cortisone palmitate (Fig. 7).

Fildes and Oliver [52] suggested that the interaction of hydrocortisone palmitate could be explained in terms of a model in which the steroid nucleus of the molecule was excluded from the hydrocarbon region of the bilayer and was associated with the polar region, whilst the ester acyl chain "dipped" into the bilayer. Knight and Shaw [55] suggested an alternative model, in which they recognised that a 21-esterified hydrocortisone molecule is hydrophobic and is unlikely to associate with the polar regions of the bilayer, but will rather penetrate into the bilayer. Arrowsmith et al. [54] have suggested that interaction between the steroid nucleus and the

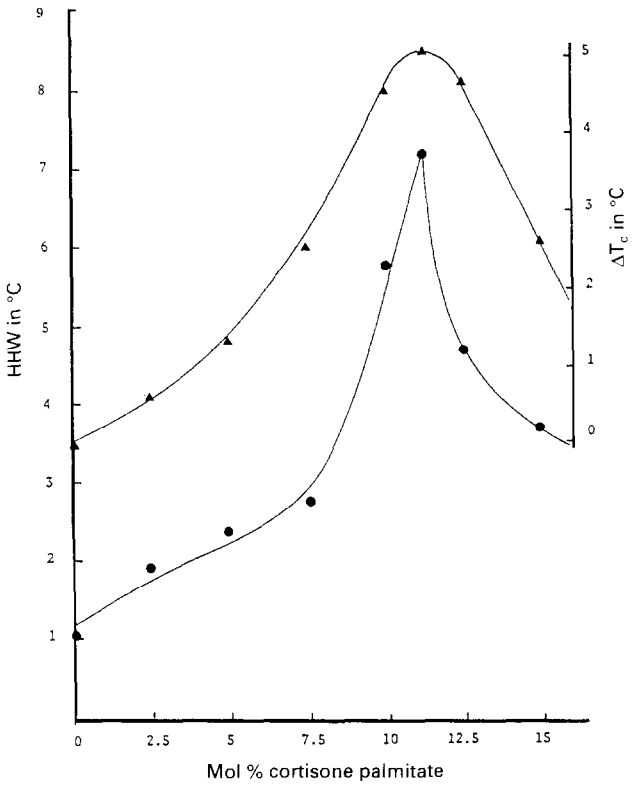

Fig. 7. The relationship between the onset of the main phase transition (T_c), the half-height width (HHW) of the endotherms and the incorporation of cortisone palmitate into DPPC Liposomes: ●, HHW; ▲, ΔT_c. (Reproduced with permission from Ref. [54].)

bilayer may make a considerable contribution to the altered transition behaviour when steroid esters are incorporated into liposomes. Conformational analysis has indicated that dexamethasone palmitate is inserted into DPPC monolayers in a similar fashion to cholesterol, with the carbonyl group orientated towards the aqueous interface and the acyl chain aligned parallel to the phospholipid hydrocarbon chains [56].

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