Chemistry and Physics of Lipids, 30 (1982) 229–259
Elsevier/North-Holland Scientific Publishers Ltd.

THE USE OF DIFFERENTIAL SCANNING CALORIMETRY AND DIFFERENTIAL THERMAL ANALYSIS IN STUDIES OF MODEL AND BIOLOGICAL MEMBRANES

RONALD N. McELHANEY

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)

Differential scanning calorimetry (DSC), and to a lesser extent differential thermal analysis (DTA), are powerful yet relatively rapid and inexpensive thermodynamic techniques for studying the thermotropic phase behavior of lipids in model and biological membranes, without the introduction of exogenous probe molecules. In this review the principles as well as the scope and limitations of DSC and DTA are discussed first. The application of these techniques to the study of the thermotropic phase behavior of aqueous dispersions of various single synthetic phospholipids are then summarized, and the effects of cholesterol, free fatty acids, lysophospholipids, drugs, anesthetics and proteins on the gel to liquid-crystalline phase transitions exhibited by these model systems are discussed. The phase mixing properties of model membranes consisting of mixtures of two or more synthetic or natural phospholipids are considered next. Finally, the thermotropic phase behavior of prokaryotic plasma membranes and of the plasma, microsomal and mitochondrial membranes of eukaryotic cells are reviewed, and the applications of DSC and DTA to study the thermal behavior of specific membrane proteins, as well as the physical properties of the membrane lipid phase, are summarized.

Keywords: differential scanning calorimetry; differential thermal analysis; lipids; biomembranes.

Introduction

Differential scanning calorimetry (DSC), and to a lesser extent differential thermal analysis (DTA), are thermodynamic techniques which have proven of great value in studies of the thermotropic behavior of lipids in model and biological membranes (for reviews, see Refs. 1–3). Historically DSC and DTA have been used primarily to study the thermally-induced transition of lipid bilayers and biological membranes from a relatively ordered, crystalline-like state existing at lower temperatures to a relatively disordered, fluid-like state at higher temperatures [1–3]. This gel to liquid-crystalline phase transition, which arises from a cooperative melting of the hydrocarbon chains of the phospholipid molecules [4–7], does not normally result in a gross molecular rearrangement to a different liquid-crystalline mesophasic state; instead, the lamellar bilayer conformation is conserved throughout [8–10]. The lipid hydrocarbon chains are converted from a relatively rigid, extended, largely all-*trans* conformation in the gel state to a more orientationally

0009-3084/82/0000-0000/$02.75

disordered state characterized by the presence of a number of *gauche* conformations and greatly increased rates of intra- and intermolecular motions [11,12]. The hydrocarbon chain melting is accompanied by a pronounced lateral expansion and a concomitant decrease in bilayer thickness, as well as by a small increase in the total volume occupied by the phospholipid molecules [8–14]. The phase transition temperature is of course markedly dependent on the chain length and the chemical structure of the lipid hydrocarbon chains, as well as on the nature of the polar head group, the interactions of which are influenced in turn by the pH and ionic composition of the aqueous phase [1–3]. The gel to liquid-crystalline phase transitions of some single, pure phospholipids appear to be sharp, symmetrical, first-order processes [15]. However, since the lipids in biological membranes typically possess appreciable fatty acid and polar head group heterogeneity, the gel to liquid-crystalline phase transitions of biomembranes are normally rather broad and often asymmetric [1–3, 7–12, 16]. Also, since lipid molecules within the bilayer undergo rapid lateral diffusion at temperatures within and above the transition temperature, fractional crystallization within the bilayer plane, with its attendant lateral phase separation, can occur [1–3,17].

The gel to liquid-crystalline lipid phase transition is certainly the most prominent and best-understood thermotropic event which occurs in model and biological membranes. Moreover, the biological effects of this transition have been relatively well studied, particularly in microorganisms [1,3,18,19]. However, there is some evidence that other lipid-mediated thermal events not directly related to the melting of the hydrocarbon chains can also occur. DSC and other physical techniques have suggested the possible presence of premelting and prefreezing phenomena, liquid-liquid phase separations, grain boundary defects in the solid state, and the glassy state in some model and biological membranes. Preliminary studies suggest that at least some of these events may influence the function as well as the structure of biological membranes (for reviews, see Refs. 1 and 3). In this brief review, however, the gel to liquid-crystalline phase transition will be emphasized. Moreover, since several excellent reviews on thermotropic events in model membranes have appeared in the mid-to-late seventies [1–3], this articles will focus primarily on the more recent literature.

Principles of DSC and DTA

The principle of DSC is comparatively simple. The sample and an 'inert' reference (a material that does not undergo a phase transition within the temperature range of interest) are simultaneously heated at identical predetermined rates. For our purposes the sample would normally be a suspension of lipid or membrane in water or an aqueous buffer, and the reference cell would contain the corresponding solvent alone. Thus the temperatures of the samples and reference cells initially increase linearly with time and the temperature difference between them is main-

tained at zero. If the sample undergoes a thermally-induced event, the control system senses the resulting temperature differential between the sample and reference cell and supplies more or less heat (power) to the sample cell to hold its temperature equal to that of the reference. The recorded parameter in DSC measurements is thus the excess specific or differential heat as a function of temperature. If the sample does not undergo a thermal event, then the recorder should trace a straight, horizontal baseline denoting a zero differential power output. If a thermal event occurs, however, the recorder pen is deflected from the baseline, the direction of the deflection depending on whether the event is endothermic (upwards) or exothermic (downwards), and the magnitude of the deflection depending on the magnitude of the differential heating rate. Upon completion of the thermal event, the recorder pen returns to the baseline or to a new baseline if a change in the specific heat of the sample has occurred.

The variation of excess specific heat with temperature for a simple two-state, first-order endothermic process, such as the gel to liquid-crystalline phase transition of a single, highly pure phosphatidylcholine, is illustrated schematically in Fig. 1. From such a DSC trace a number of important parameters can be directly determined. The phase transition temperature, usually denoted T_m or T_c, is that temperature at which the excess specific heat reaches a maximum. For a symmetrical curve, T_m represents the temperature at which the transition from the gel to liquid-crystalline state is one-half complete. However, for asymmetric traces which are characteristic of certain pure phospholipids and many biological membranes, the T_m does not represent the midpoint of the phase transition, and a $T_{1/2}$ value may be reported instead. The peak area under the DSC trace when calibrated with a known standard is a direct measurement of the calorimetrically determined enthalpy of the transition, ΔH_{cal}, usually expressed in kcal/mol. The area of the

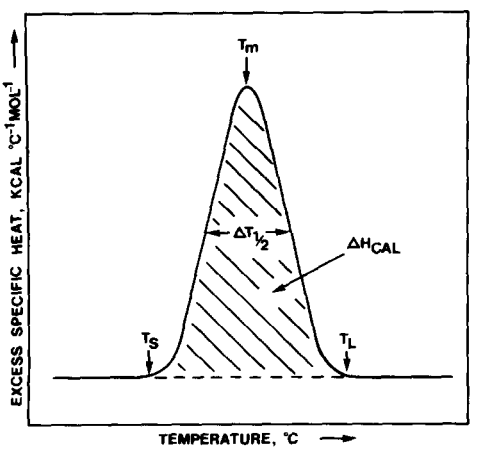

Fig. 1. The variation of excess specific heat with temperature during a two-state, endothermic process. The symbols are explained in the text.

peak can be determined by planimetry or by the cutting and weighing technique; alternatively, the calorimeter output can be digitized and the T_m and ΔH_{cal} calculated by a computer. Since at the phase transition midpoint-temperature the change in free energy (ΔG) of the system is zero, the entropy change associated with the transition can be calculated directly from the equation

$$\Delta S = \Delta H_{cal}/T_m$$

where ΔS is normally expressed in cal/K · mol.

The sharpness or cooperativity of the gel to liquid-crystalline phase transition can also be evaluated from the DSC trace. The sharpness of the phase transition is often expressed as the temperature width at half-height, $\Delta T_{1/2}$, or as the temperature difference between the onset or lower boundary of the phase transition, T_s, and the completion or upper boundary, T_l, or $\Delta T = T_l - T_s$. The $\Delta T_{1/2}$ values may range from $<0.1^{\circ}C$ for very pure synthetic phospholipids to as much as 10–15°C for biological membranes. From the T_m and $\Delta T_{1/2}$ values determined for a particular phase transition, the van't Hoff enthalpy, ΔH_{vH}, can be approximately determined from the relationship

$$\Delta H_{vH} \simeq 4RT_m^2/\Delta T_{1/2}$$

From the ratio $\Delta H_{vH}/\Delta H_{cal}$, the cooperative unit (CU) (in molecules) can be determined [2]. The CU is a measure of the degree of intermolecular cooperation between phospholipid molecules in a bilayer; for a completely cooperative, first-order phase transition of an absolutely pure substance, this ratio should approach infinity, while for a completely non-cooperative process this ratio should approach unity. Although the absolute CU values determined should be regarded as tentative, since this parameter is markedly sensitive to the presence of impurities and may be limited by instrumental parameters, carefully determined CU values can be useful in assessing the purity of synthetic phospholipids and in quantitating the degree of cooperativity of lipid phase transitions.

Differential thermal analysis is superficially related to DSC although the measured parameter is different. In DTA both the sample and inert reference are heated together in a single block at some constant, predetermined rate. In this case, however, the temperature differential, as opposed to the differential heat flow, is recorded as a function of the temperature of the reference material. As in DSC, the DTA thermogram provides accurate, comparable information on the transition temperature, the temperature range over which the transition occurs, and on whether the transition is endothermic or exothermic. Also, in principle the peak area should be related to the enthalpy of the transition. However, because the DTA thermogram peak area is also sensitive to the heat capacity, thermal diffusivity and geometry of the sample, it is in practice difficult to calibrate the system and thus to determine accurate enthalpy values. DSC minimizes the dependence of peak area upon extraneous factors since the heat delivered to the entire

sample is measured directly rather than as the temperature differential at some point within the sample. Although DTA was often employed in early studies because commercial DTA instruments were more sensitive and stable than the low-sensitivity DSC units then available, the present availability of high-sensitivity DSC instruments makes DSC the technique of choice.

It must be stressed that for valid thermodynamic parameters to be obtained by DSC, the system being studied must be at equilibrium throughout the calorimetric run. Although it appears that the gel to liquid-crystalline phase transition of certain single, pure synthetic phosphatidylcholines is quite rapid and not therefore kinetically limited even at moderate scan rates [15], this may well not always be the case. It has been demonstrated, for example, that the 'pretransition' of synthetic disaturated phosphatidylcholines is kinetically limited even at the relatively slow scan rates obtainable with high-sensitivity DSC instruments [20, 21, Lewis et al., unpublished data]. It is thus important to establish that the thermodynamic parameters being measured are not significantly dependent on scan rate, either due to a rate limitation of the process being studied, or to thermal lags or instrumental response problems in the calorimeter itself. It should be remembered that any system at equilibrium must proceed in an endothermic direction when the temperature is increased. Heat can only be evolved with increasing temperature if the process under study is kinetically rather than thermodynamically limited.

DSC is probably the single most powerful technique for the routine measurement of gel to liquid-crystalline phase transitions in lipid bilayers and biological membranes. The instrumentation required is comparatively inexpensive, the measurements can normally be made relatively quickly, and the interpretation of the data obtained is fairly straightforward. Also the current high-sensitivity DSC instruments require only relatively dilute suspensions of material permitting accurate control of the pH and ionic composition of the aqueous phase. Unlike some other techniques which have been utilized to study lipid thermotropic behavior, DSC accurately reports the entire course of broad phase transitions including the onset and completion temperatures as well as the general shape of the phase transition. Moreover, unlike a number of spectroscopic techniques, DSC does not require the introduction of foreign probe molecules into the system under study; this is a distinct advantage since evidence is accumulating that these probes may localize at phase boundaries and can cause microenvironmental perturbations [3,22–27]. Finally, this technique is unique in providing a direct and accurate measurement of the thermodynamic parameters of the lipid phase transition under investigation. It should be remembered, however, that a thermodynamic technique such as DSC does not provide direct information about molecular structure or dynamics. However, the combination of DSC with a direct structural technique such as X-ray or neutron diffraction, or with an appropriate non-perturbing spectroscopic technique such as nuclear magentic resonance (NMR) spectroscopy, provides a very powerful approach to understanding thermal events in membrane systems at the molecular level.

DSC and DTA studies of model membranes

Studies of single phospholipids

When a pure phospholipid is dispersed in water by mechanical agitation, most form closed spherical structures composed of a number of concentric bilayer sheets separated by aqueous spaces. These multilamellar suspensions of phospholipids (liposomes) usually exhibit one or more highly cooperative, reversible thermal phase transitions. The phosphatidyl cholines (PCs) containing two identical, even-chain saturated fatty acyl groups have been the most intensively studied by DSC and other techniques. A high-sensitivity DSC trace of a multilamellar suspension of 1,2-dipalmitoyl phosphatidylcholine (DPPC) is presented in Fig. 2. The major chain-melting transition is characterized by a sharp, symmetric, relatively energetic, first-order endothermic transition. A second, broader endothermic transition of lower enthalpy occurs about 7°C below the gel to liquid-crystalline transition, the distance between this pretransition temperature (T_p) and the main transition (T_m) appearing to decrease with increasing chain length, with the $T_p \cong T_m$ for a chain length of 22 carbons. We have recently established, however, that the observed T_p for a series of simple disaturated PCs is markedly scan rate-dependent, progressively more so for PCs with chain lengths above 16 carbons; thus the T_p values currently reported for these compounds are considerably higher than the true equilibrium values, particularly for the longer chain length PCs. A plot of T_p values extrapolated to zero scan rate versus the chain length of the PCs reveals that the $\Delta T_m - T_p$ reaches a local minimum at 17 carbons and increases slightly at longer

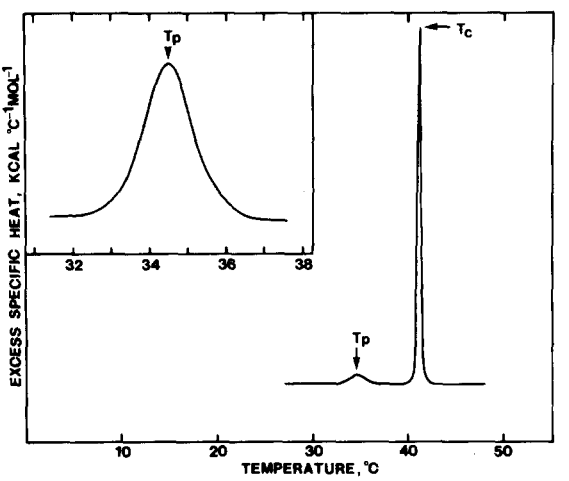

Fig. 2. A typical high-sensitivity DSC trace of a multilamellar, aqueous suspension of dipalmitoyl phosphatidylcholine. The pretransition and main transition temperatures are denoted by T_p and T_m, respectively. The inset shows the pretransition on expanded excess specific heat and temperature scales.

chain lengths (Lewis et al., unpublished data). PCs containing saturated fatty acyl chains of less than 14 carbons do not exhibit the pretransition. Also, the pretransition is abolished by small amounts of cholesterol, fatty acids or other compounds [2]. Interestingly the T_p values at least of the medium-chain PCs exhibit an odd-even alternation while the T_m values fall on a single smooth curve [28, Lewis et al., unpublished data].

The thermodynamic parameters associated with the pretransition and main transition of a series of simple disaturated PCs have been determined by a number of workers (see Ref. 2, Lewis et al., unpublished data). The pretransition is characterized by ΔH_{cal} values of about 1.0–1.8 kcal/mol and ΔH_{cal} does not vary markedly with chain length. The CU of this transition is relatively large, typically of the order of several hundred molecules, comparable to values for the main transition; the magnitude of the CU does not appear to be markedly chain length-dependent. The main transition is characterized by ΔH_{cal} values which increase progressively with chain length from about 1.7 kcal/mol for dilauroyl PC (DLPC) to about 10.6 kcal/mol for distearoyl PC (DSPC); ΔS values also increase progressively with increasing chain length [4]. A linear relationship between ΔH_{cal} and T_m has been reported for the series of even-chain PCs from 12 to 22 carbons [2]. However, in our hands such a plot is not linear and ΔH_{cal} values tend to plateau at the longer chain lengths (Lewis et al., unpublished data). The CU values reported for the main transition range from several hundred to over a thousand molecules [2,15], these differences probably being due largely to differences in the relative purities of the samples studied. The CU value of the main transition also does not appear to be markedly chain length-dependent.

The thermotropic phase behavior of simple diacyl PCs containing two identical *n*-saturated, methyl *iso*- or *anteiso*-branched saturated, or *cis*- and *trans*-monounsaturated and *cis*- and *trans*-cyclopropyl fatty acyl groups of several different chain lengths has recently been studied by DTA [29–31]. The T_m values of the 18-carbon members of this series of PCs decreased in the following order: *n*-saturated (54.8°C) > methyl *iso*-branched (36.5°C) > methyl *anteiso*-branched (18.7°C) ≃ *trans*-cyclopropane (16.3°C) > *trans*-mono-unsaturated (12.9°C) > *cis*-cyclopropane (−0.5°C) > *cis*-mono-unsaturated (−15.8°C). The effect of the introduction of a *cis*-double bond or *cis*-cyclopropyl group on the T_m of simple diacyl PCs has recently been confirmed by DSC [32]. The correspondence between the melting points of the anhydrous free fatty acids and the T_m values of the corresponding fully hydrated PC bilayer dispersion is only fair. In contrast to the di-*n*-saturated PCs, no pretransitions were observed for the branched chain, unsaturated or cyclopropane fatty acid-containing PCs; moreover, the methyl *iso*- and *anteiso*-branched PCs exhibited an odd-even alternation in their T_m values indicating that their fatty acyl chains are tilted in the gel state. An increase in chain length from 16 to 18 carbons increases T_m by 13.3°C and 14.5°C for the *n*-saturated and methyl *iso*-branched, by 16.6°C and 16.9°C for the *trans*-mono-unsaturated and cyclopropyl, by 19.4°C and 19.7°C for the *cis*-mono-unsaturated and cyclopropyl, and by

21.7°C for the methyl *anteiso*-branched PCs. Although accurate ΔH_{cal} values could not be obtained in these DTA studies, it appeared that the ΔH_{cal} values were reduced in the methyl *iso*-branched and particularly in the *anteiso*-branched PCs as compared to their *n*-saturated analogs, suggesting the existence of a less ordered gel phase. This conclusion has recently been partially supported by the results of an X-ray diffraction study [33]. Accurate ΔH_{cal} and CU values for these series of PCs are currently being obtained by high-sensitivity DSC.

Multilamellar suspensions of diphytanoyl PC are unusual in exhibiting no calorimetrically detectable gel to liquid-crystalline phase transition over the temperature range −120°C to +120°C. NMR studies revealed that this unusual PC undergoes a gradual decrease in orientational order and a gradual increase in the rates of molecular motion over a wide temperature range. It appears that the presence of multiple methyl branches along the hydrocarbon chains maintains this PC in an expanded and only partially ordered state even at very low temperatures [34].

The thermotropic behavior of mixed-acid saturated PCs containing myristate, palmitate or stearate chains has recently been investigated by conventional [35] and high-sensitivity DSC [36,37]. In each study the interesting observation was made that the T_p, T_m and ΔH_{cal} values of each isomeric pair of PCs were different, with the isomer having the longer chain at position 1 of *sn*-glycerol exhibiting significantly lower T_p, T_m and ΔH_{cal} values. When the fatty acid chain length in position 2 is constant, increasing the chain length at position 1 will increase T_p and T_m, but will not alter the ΔH_{cal} of the main transition. On the other hand increasing the chain length at position 2 with a constant chain length at position 1 also increases T_p and T_m, but in addition, also increases ΔH_{cal} by about 0.5 kcal/mol for each CH segment added, just as previously observed for saturated PCs with identical acyl chains. These results can be rationalized by examining the structure of PCs in bilayers in both the gel and liquid-crystalline states. Both NMR [38–40] and neutron [41] and X-ray [42,43] diffraction studies have shown that identical acyl chains at the 1- and 2-positions of the glycerol backbone nevertheless have different conformations. The acyl chain at position 2 begins roughly parallel to the bilayer surface before bending at C2 to orient the hydrocarbon chain perpendicular to the bilayer plane, whereas the fatty acyl chain at position 1 is perpendicular to the bilayer throughout its length; thus, the effective length of the fatty acyl chain at the 1-position is about 1.8–1.9 Å or about 1.5 C–C bond lengths longer than the same fatty acid esterified to position 2. Therefore, saturated mixed-acid PCs with the shorter chain acid at position 1 tend to minimize the intrinsic chain length mismatch due to the differing conformations of the two fatty acyl chains, resulting in a more stable bilayer with a higher T_m and ΔH_{cal} than is the case for the corresponding positional isomer in which the intrinsic chain length mismatch is in fact accentuated. Interestingly, all the mixed-acid saturated PCs isomers examined exhibit a pretransition except 1-myristoyl-2-stearoyl PC. Also, the pretransition ΔH_{cal} values for all positional isomers examined are lower than those reported for saturated PCs containing identical fatty acids, except for the isomer 1-stearoyl-2-myristoyl PC which has a considerably higher ΔH_{cal}.

The thermotropic behavior of several mixed-acid saturated-unsaturated PCs containing oleic and elaidic acids have been examined by DSC and DTA [6,44–48]. For 1-palmitoyl-2-oleoyl PC, reported T_m values range from −5°C to +3°C and ΔH_{cal} values from 8.0 to 8.1 kcal/mol; comparable values for the reversed isomer 1-oleoyl-2-palmitoyl PC are −11°C and 6.7 kcal/mol, qualitatively the results expected from a consideration of the differential effective chain length effect just discussed. It is noteworthy that the T_m values for these mixed-acid, saturated-unsaturated PCs fall well below the average for the corresponding disaturated and di-unsaturated compounds ($T_m \cong 41$°C and −14 to −22°C for DPPC and dioleoyl PC (DOPC), respectively [49]). On the other hand, the ΔH_{cal} for 1-palmitoyl-2-oleoyl PC (8.0 kcal/mol) falls near the average for DPPC and DOPC (about 8.5 kcal/mol and 7.6 kcal/mol, respectively [49]), while the corresponding value for 1-oleoyl-2-palmitoyl PC (6.7 kcal/mol) is below that of DOPC. In contrast, the T_m of 1-palmitoyl-2-elaidoyl PC (35°C) lies closer to the T_m of DPPC than to that of dielaidoyl PC (DEPC) (9.5–13°C [49]); unfortunately, a ΔH_{cal} value for this lipid is not available, nor has the reverse isomer been studied. For 1-stearoyl-2-oleoyl PC, reported T_m values range from +3°C to 13°C and ΔH_{cal} values from 4.3 to 5.3 kcal/mol; a T_m value is not available for 1-oleoyl-2-stearoyl PC, but a ΔH_{cal} of 6.7 kcal/mol has been reported. Again, the T_m values fall well below the average for the corresponding disaturated and di-unsaturated analogs ($T_m \cong 54$°C for DSPC) and the ΔH_{cal} values fall well below those of DOPC. Interestingly, the T_m for 1-stearoyl-2-elaidoyl PC (26°C) falls closer to that of DEPC than to DSPC and well below the T_m of 1-palmitoyl-2-elaidoyl PC, despite its greater average chain length, again illustrating the effect of the different chain conformations at positions 1 and 2; the ΔH_{cal} for 1-stearoyl-2-elaidoyl PC (8.4 kcal/mol) again falls below the average for DSPC and DEPC (ΔH_{cal} for DEPC = 7.3–10.0 kcal/mol, [49]). It thus appears that in the absence of intrinsic chain length mismatch compensation, the gel state stability of at least the mixed-acid saturated-*cis*-unsaturated PCs more closely resembles that of the corresponding di-unsaturated than that of the disaturated analogs. Also the mixed-acid saturated-unsaturated PCs, like the di-unsaturated species, do not exhibit pretransitions.

The thermotropic behavior and other physical properties of 1,3-DPPC have recently been studied by DSC, NMR and monolayer techniques and compared to those of the naturally occurring isomer 1,2-DPPC [50]. The T_p and T_m of the 1,3-isomer were reduced by about 15°C and 3–4°C, respectively, compared to the 1,2-isomer, whereas the ΔH_{cal} of the main transition was increased by about 1 kcal/mol. The 1,3-DPPC formed more expanded monolayer films at all pressures and the hydrocarbon interiors of the bilayers were more disordered than for 1,2-DPPC. In 1,3-DPPC bilayers both fatty acyl chains were found to begin parallel to the bilayer surface, and are bent perpendicular to it after the C-2′ segment. The effective lengths of both palmitoyl chains are thus shortened, presumably resulting in reduced intermolecular interactions which would account for the lower T_p and T_m values of the 1,3-isomer, for its expanded cross-sectional area and for its more disordered hydrocarbon chains. The basis for the increased heat content of the

main transition of the 1,3-isomer is, however, unclear.

The thermotropic behavior of the complete series of positional isomers of di-*cis*-octadecenoyl PCs has been studied by DSC and DTA [51]. A plot of T_m versus double bond position fell on a smooth U-shaped curve with a minimum at the center of the hydrocarbon chain. T_m values ranged from +45°C and +41°C for the Δ17 and Δ2 isomers, respectively, to −21°C for the Δ9 and Δ10 isomers. The ΔH_{cal} values were also higher when the double bond was located near either end of the chain (9.6 kcal/mol) than near the chain center (7.5–7.7 kcal/mol); ΔS values did not vary significantly with double bond position. A similar variation in T_m with double bond location was observed for the more limited number of 1-stearoyl-2-*cis*-octadecanoyl PC isomers examined. Interestingly, of the sixteen positional isomers of di-*cis*-octadecenoyl PC studied, only the Δ15 and Δ16 isomers (and possibly the Δ17 isomer) exhibited a pretransition.

The effect of the presence of a number of alkaline, alkaline earth and heavy metal salts on the phase behavior of PCs have been investigated by DSC and DTA [52–55]. The T_p and T_m values, and their corresponding ΔH_{cal} values, were little affected by monovalent cations including Na^+ and K^+, even at concentrations of 1 M. The presence of 1 M Mg^{2+} increased T_p, T_m and ΔH_{cal} for the main transition slightly, while Ca^{2+} concentrations of >10 mM resulted in a substantial increase in T_p and a less marked increase in T_m, until the two transitions merged at Ca^{2+} concentrations >250 mM; increasing Ca^{2+} concentration above 1 mM also reduced the ΔH_{cal} of the pretransition, but increased this value at concentrations above 10 mM. The thermotropic behavior of the zwitterionic PCs are generally much less sensitive to the presence of cations than are the negatively charged phospholipids.

In multilamellar dispersions of phospholipids having identical fatty acyl chains, the nature of the polar headgroup is also important in determining thermotropic behavior. Calorimetric studies of a number of phosphatidylethanolamines (PEs) at pH values near neutrality have been reported [53,56–61]. Simple disaturated PEs do not exhibit pretransitions and the main transition is markedly asymmetric, in contrast to the simple disaturated PCs. The T_m values of the disaturated PEs are always considerably greater than for the corresponding PCs, the ΔT_m being greatest for the dilauroyl species (~30°C) and progressively decreasing as the chain length increases (ΔT_m for DSPE and DSPC ≅ 17°C). Although the T_m for DEPE (35–38.5°C) is also considerably higher than for DEPC (9.5–13°C), the T_m values for dipalmitoleoyl PE (−33.5°C) and DOPE (−16°C) are only slightly higher than for dipalmitoleoyl PC (−35.5°C to −36°C) and DOPC (−14°C to −22°C). The ΔH_{cal} values for the disaturated PEs, however, are comparable to those of the corresponding disaturated PCs, while the ΔH_{cal} values for the di-unsaturated PEs are only about half as large as for the corresponding PCs. The CUs for the disaturated PEs are also only about half as large as those of the corresponding disaturated PCs [62]. The increased stability of disaturated PE in the gel state appears to be due to both the smaller size of the PE as opposed to the PC head group [62], and to the ability of the protonated amino group of PE to hydrogen bond to the pro-

tonated amino and negatively charged phosphate groups of adjacent PE molecules [61,63]. The observation that the deprotonation of the DPPE amino groups, by increasing the pH to 12, decreases T_m from 63°C to 41°C (the T_m for DPPC) supports the latter view [64]. At pH < 8 synthetic and naturally occurring unsaturated PEs undergo a sharp, bilayer-to-hexagonal (H_{11}) phase transition at a temperature above [58], or sometimes within [61] the gel to liquid-crystalline temperature range of these PEs. The enthalpy of this transition is low and it cannot always be detected by conventional DSC.

Calorimetric studies of the negatively charged phospholipid phosphatidylglycerol (PG) have been carried out and the effect of variations in fatty acyl group composition, pH and ionic environment on the thermotropic behavior of this compound have been investigated [66–70]. When dispersed in water at neutral pH, disaturated PGs have T_m, ΔH_{cal} and ΔS values almost identical to the corresponding PCs; moreover, these disaturated PGs also exhibit a pretransition which is lacking in the di-unsaturated analog DOPG. Also like the corresponding PCs, PG thermotropic behavior is only slightly affected by the nature and concentration of monovalent cations present in the aqueous phase. However, in the presence of Ca^{2+} or Mg^{2+} PG forms a high-melting, metastable complex in contrast to the PCs. The phase behavior of the PGs is also markedly dependent on the pH and thus the charge of the polar head group. At pH 2, the T_m of DPPG increases from 41°C (pH 7) to 61°C, which has been ascribed to a reduction of the repulsive forces between adjacent negatively charged phosphate groups. Interestingly, partial protonation of the phosphate of PC has a similar effect on its thermotropic behavior despite the presence of a positively charged quaternary amine group. The similarity in the thermotropic behavior of PG and PC has led to the suggestion that PC behaves in effect as a negatively charged rather than a zwitterionic lipid at neutral pH, its behavior being little affected by the presence of its positively charged but shielded amine moiety [64].

The phase transition characteristics of diphosphatidylglycerols (cardiolipins, CL) and their dependence on ionic environment have recently been studied by DSC [71]. In general, their thermotropic behavior (T_m and ΔH_{cal} values per chain) is very similar to that of the corresponding PGs and PCs, and a pretransition occurring some 5–15°C below the main transition and having an enthalpy about 10% of that of the main transition is also observed. The T_m of metal salts of DPCL was found to exhibit a biphasic dependence on the unhydrated ionic radius, with the T_m being highest for DPCL-Ca^{2+} and decreasing for ions of either smaller or larger radii.

The phase behavior of natural and synthetic disaturated phosphatidylserines (PSs), and how this behavior is influenced by pH and cation concentration, has been investigated by several groups utilizing DSC and other physical methods [66,68,72]. At neutral pH, where the PS molecule bears two negative and one positive charge, the disaturated PSs exhibit T_m values considerably higher than observed for the corresponding PCs, PGs or CLs, but lower than for the corresponding PEs. The ΔH_{cal} values are similar to those of the other phospholipids discussed and a pretransition does not occur. At low pH where the free carboxyl group of

DPPS is protonated and the molecule is thus zwitterionic, the T_m is elevated to 72°C and the ΔH_{cal} to about 9 kcal/mol; at higher pH where the carboxyl group becomes fully ionized, T_m falls to 55°C and ΔH_{cal} to about 3 kcal/mol. While the increase in T_m and ΔH_{cal} upon protonation of the carboxyl group of PS can be qualitatively explained by a diminution of the repulsive forces acting between the negatively charged headgroups at neutral pH, the relatively high T_m of this phospholipid suggests that, as for PEs, intermolecular hydrogen bonding makes a major contribution to bilayer stability. Although the phase behavior of PS is rather insensitive to the nature and concentration of the monovalent cations present, Ca^{2+} and to a lesser extent Mg^{2+} form tightly packed, water-excluding, quasi-crystalline complexes with PS which do not melt below 100°C.

Although present in biological membranes in only trace amounts, the thermotropic phase behavior of phosphatidic acid (PA) as a function of pH has been studied extensively by DSC and other techniques [66,68,73–75], since this phospholipid is unique in having two dissociable protons on its phosphate group. At neutral pH where the disaturated PAs have a net negative charge of one, these compounds exhibit T_m values which are even higher than those of the corresponding PEs although the ΔH_{cal} values are similar to those of PE and of the other phospholipids. The high T_m of PA, despite its negative charge at pH 7, can also be explained by intermolecular hydrogen bonding between adjacent molecules since at this pH one dissociable proton is still present. As the pH is lowered, the T_m actually increases slightly, reaching a local maximum near pH 4; at this pH, the first proton is only 50% dissociated and hydrogen-bonding opportunities are maximized. A further decrease in pH resulting in complete protonation of PA, results in a decrease in T_m of about 9°C and also a marked reduction in ΔH_{cal} values. Similarly, when the second phosphate proton is completely dissociated at pH 11, the T_m also decreases drastically, by about 25°C, as does the ΔH_{cal}, presumably due to the repulsion of the doubly negatively charged phosphate head groups and their inability to form hydrogen bonds with adjacent PA molecules. Interestingly, at high pHs disaturated PAs exhibit a pretransition as do the disaturated PCs and PGs at neutral pHs. Recent evidence indicates that the pretransition exhibited by these phospholipids results from a rearrangement of the lattice packing of the hydrocarbon chains from a planar bilayer, in which the chains are tilted with respect to the bilayer plane, to a rippled bilayer in which the bilayer plane is locally everywhere curved and the hydrocarbon chain tilt is altered or abolished [28, Lewis et al., unpublished data]. In order to study the thermotropic behavior of these phospholipids at extremes of pH the diether rather than the diester forms were utilized, since the former are resistant to hydrolysis of the fatty acyl chains at acidic and basic pHs. The diether phospholipids generally have similar but not identical T_m and ΔH_{cal} values to the corresponding diester compounds [56, 73–75]. The reader is referred to an excellent recent review for a fuller discussion of the effect of intermolecular hydrogen bonding on the properties of lipids in model and biological membranes [76].

The thermotropic behavior of small single bilayer vesicles of a particular phos-

pholipid differs significantly from that of the same lipid in a multilamellar dispersion. The phase behavior of small phospholipid vesicles prepared by ultrasonic irradiation has been studied by DSC in conjunction with other physical techniques [77–81]. It was initially reported that the calorimetric characteristics of sonicated and unsonicated lipid dispersions were indistinguishable by conventional DSC, and later that sonicated disaturated PC vesicles exhibited a less enthalpic pretransition and a downward-shifted, somewhat broadened main phase transition with only a slightly reduced ΔH_{cal} [80]. However, studies employing high-sensitivity DSC demonstrated that in freshly sonicated vesicles of disaturated PCs the pretransition is absent and that the ΔH_{cal} of the main transition is drastically reduced [78,79, 81]. The broader thermotropic phase transition exhibited by the sonicated vesicles was shown to result mostly from the reduced ΔH_{cal} rather than from a marked decrease in the CU [81]. The results obtained with conventional DSC were probably due to a time-dependent aggregation of unilamellar vesicles into larger multilamellar vesicles promoted by the high lipid concentrations required. Also vesicle fusion was shown to be enhanced by cycling through the lipid phase transition or by the freezing of the aqueous phase, as well as by an increased medium osmolarity, the addition of fatty acids, or the presence of divalent cations such as calcium [79, 80]. A number of other physical techniques have indicated that the small radius of curvature of sonicated phospholipid vesicles leads to a less ordered orientation and to a greater freedom of motion of the phospholipid hydrocarbon chains than are found in larger vesicles [1–3].

Studies of phospholipid mixtures

Although studies of the thermotropic phase behavior of single-component multilamellar phospholipid vesicles are necessary and valuable, these systems are not realistic models for biological membranes which normally contain at least several different types of phospholipids and a variety of fatty acyl chains. As a first step toward understanding the interactions of both the polar and apolar portions of different lipids present in mixtures, DSC studies of various binary phospholipid systems have been carried out. Phase diagrams can be constructed by specifying the onset and completion temperatures for the phase transition of a series of mixtures and by an inspection of the shapes of the calorimetric traces. A comparison of the observed transition curves with the theoretical curves supports a literal interpretation of the phase diagrams obtained by DSC [2].

The thermotropic behavior of mixtures of two PCs differing in the nature of their fatty acyl constituents has been investigated. Disaturated PCs differing by only two carbons in the length of their hydrocarbon chains exhibit almost ideal behavior in binary phospholipid-water dispersions (see Fig. 3). Although a difference in chain length of four carbons presents a system considerably removed from ideality, isothermal melting of the shorter-chain PC is not observed and a significant degree of lateral phase separation does not occur (but see Ref. 83). A difference in hydrocarbon chain length of six carbons results in monotectic behavior, with the chain-melting onset temperature remaining constant over most of the concentration

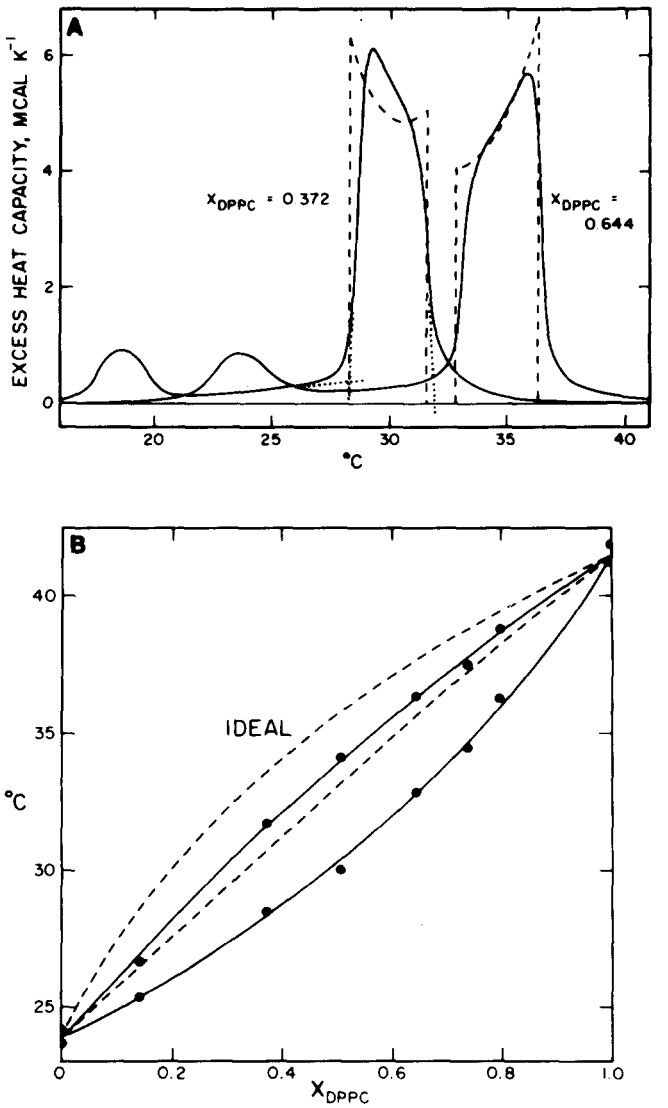

Fig. 3. (A) ———, observed calorimetric transition curves for two mixtures of DMPC and DPPC. – – – –, transition curves calculated on the basis of the ideal phase diagram in B. (B) ———, phase diagram constructed from initiation and completion temperatures derived from the observed transition curves. – – – –, the ideal phase diagram. (Reproduced from Mabrey and Sturtevant (1978) [2] with permission from Plenum Press).

range; a region of pronounced lateral phase separation is evident from the DSC traces (see Fig. 4) [46,52,57,82,83]. Monotectic behavior is also observed for the systems DOPC plus DMPC, DPPC or DSPC, indicating that di-*cis*-mono-unsaturated PCs and disaturated PC of whatever chain length are largely immiscible in the gel state [82]. In contrast, DEPC and DMPC are nearly perfectly miscible in all propor-

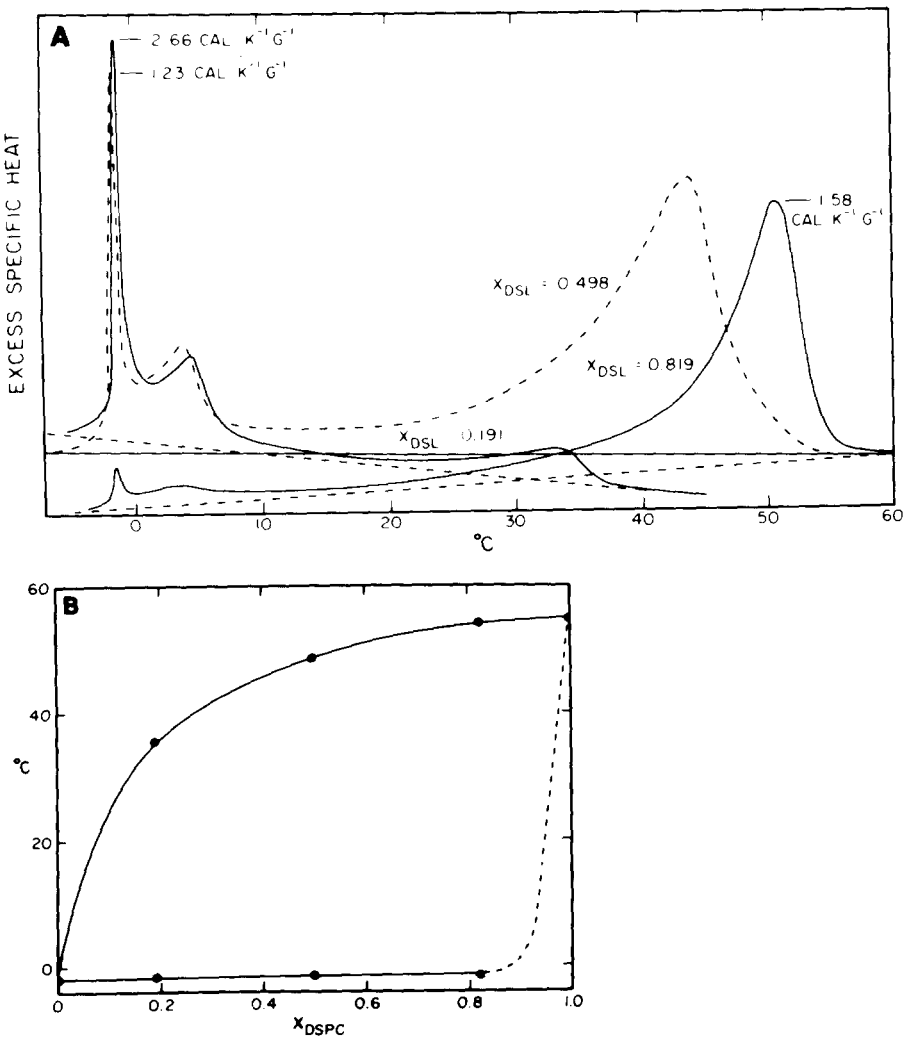

Fig. 4. (A) ———, dashed baselines: observed calorimetric transition curves for DLPC–DSPC mixtures of mole fraction DSPC (X_{DSPC}) = 0.191 and 0.819. – – – –, solid baseline: observed calorimetric curve for X_{DSPC} = 0.498. (B) Phase diagram constructed from the calorimetric transition curves. (Reproduced from Mabrey and Sturtevant (1978) [2] with permission from Plenum Press).

tions, while DEPC and DPPC mix less ideally and exhibit a solid-solid immiscibility gap at mole fractions of DPPC from about 0.30 to 0.55 [83]. In general, the smaller the difference in the T_m values of these simple diacyl PCs, the more nearly ideal is their mixing behavior. Phase diagrams for the binary systems 1-palmitoyl-2-oleoyl PC : DPPC and 1-stearoyl-2-oleoyl PC : DPPC have also been constructed using DSC [47]. The POPC–DPPC system exhibited behavior that was far from ideal but there was little gel-state immiscibility at any composition, whereas the SOPC–DPPC system exhibited appreciable gel state immiscibility particularly at DPPC concentrations of less than 50 mol%. Of these latter two binary systems, the more nearly ideal behavior was exhibited by the PC pair with the largest difference in T_m values, indicating that the miscibility of PCs in bilayers may be influenced by more subtle structural variations as well as by differences in chain-melting temperatures.

A number of calorimetric studies of binary mixtures of two different phospholipids containing either similar or dissimilar fatty acids have been done. For binary mixtures of disaturated PG–Na^+ and PCs with identical acyl chains nearly ideal mixing was observed. Mixing simple PGs and PCs with increasingly large differences in the chain lengths of their saturated fatty acyl groups, or mixing disaturated with diunsaturated lipids, produced increasingly non-ideal behavior but no more so than for a binary mixture of PCs containing the same fatty acids. These results indicate a high degree of miscibility of the PG and PC headgroups either in the presence or absence of Ca^{2+}. Interestingly, small amounts of PC were found to abolish the formation of high-melting metastable PG–Ca^{2+} or PG–Mg^{2+} complexes observed with the pure PGs alone [69].

Several groups have studied binary mixtures of PC and PE [52,84,85]. Mixtures of these two phospholipids having identical fatty acyl groups, such as DMPC–DMPE, exhibited quite non-ideal behavior. Although these lipids seemed miscible in the liquid-crystalline phase, the solidus curves of these phase diagrams showed a minimum at about 20 mol%, indicating gel state immiscibility in this composition range. Some gel-state immiscibility was also observed in DMPE–DPPC and DMPE–DSPC mixtures; however, those mixtures, particularly the latter one, exhibited more nearly ideal behavior than did the DMPE–DMPC system. Since the ΔT_m values also decreased as the chain length of the PC component of the DMPE–PC mixtures increased from 14–18 carbon atoms, these results suggest that the relative chain-melting temperatures rather than the absolute relative chain lengths are of primary importance in determining PE–PC miscibility. Nevertheless, even when the T_m values of both components are closely matched, PE–PC mixtures display considerably more non-ideality than comparable PC–PC or PC–PG mixtures, indicative of some polar head group immiscibility.

The phase behavior of mixtures of DPPC with bovine brain PS [66,86] has been studied by DSC in conjunction with other techniques. Complex and somewhat non-ideal phase mixing occurs, but more quantitative conclusions about the miscibility of polar head groups are difficult since the natural PS is already a

mixture of molecules differing in the length and degree of unsaturation of their fatty acyl chains. Calorimetric studies of egg PC-cerebroside [87] and synthetic PC-ganglioside [48,88] mixtures have also been published. For a summary of calorimetric and other studies of the thermotropic phase behavior of natural and synthetic sphingomyelins (SMs) and of their mixing properties with cholesterol and phospholipids, the reader is referred to a recent comprehensive review [89].

Recent theoretical analyses of the phase diagrams of binary mixtures of disaturated PCs of different chain lengths and of PC–PE mixtures, indicates that microscopic mixing may be significantly non-ideal in the liquid crystalline as well as the gel phases, even though no macroscopic lateral phase separations occur [90,91]. Thus, microscopic clusters of like lipid molecules may exist in the gel and liquid-crystalline phases although generally more random mixing will occur in the liquid-crystalline phase. Interestingly, the propensity for the self-association of two different liquids in the gel and liquid-crystalline states is not always related, neither is this propensity always correlated with the macroscopic phase behavior of that particular system. Some indirect non-calorimetric evidence for phospholipid clustering in the liquid-crystalline state is available (see Ref. 76 for a review).

Because alterations in pH and ionic concentration can cause significant changes in the T_m particularly of acidic phospholipids, isothermal phase transitions can be induced in pure phospholipids by variation in pH or divalent cation concentration. Similarly an isothermal lateral phase separation in liquid-crystalline mixtures of a zwitterionic lipid such as PC and an acidic lipid such as PG, PS or PA, can also be induced by changes in pH or by the addition of Ca^{2+}; in this case domains of gel or quasi-crystalline acidic phospholipids can presumably be formed, leaving the neutral phospholipid in separate fluid domains. Calorimetric and other evidence for Ca^{2+}-induced phase separations in PS–PC [66,68,92–94], PA–PC [66,95–97] and PG–PC [67–69] mixtures has been published. Apparently Mg^{2+} is not as effective at inducing lateral phase separations in such binary mixtures [66,93,97,98].

Effect of cholesterol on the thermotropic behavior of phospholipids

The occurrence of cholesterol and related sterols in the membranes of eukaryotic cells has prompted many investigations of the effect of cholesterol on the thermotropic phase behavior of phospholipids [1–3]. Studies utilizing calorimetric and other physical techniques have established that cholesterol can have profound effects on the physical properties of phospholipid bilayers and probably plays an important role in controlling the fluidity of biological membranes (for a review, see Ref. 99). Cholesterol induces an 'intermediate state' in phospholipid molecules with which it interacts, increasing the fluidity of the hydrocarbon chains below, and decreasing the fluidity above the gel to liquid-crystalline phase transition temperature.

A number of groups have studied PC–cholesterol mixtures using conventional DSC [1–3]. Although there was general agreement that the presence of increasing concentrations of cholesterol broadened the gel to liquid-crystalline phase transi-

tion of PC and reduced its ΔH_{cal}, there was disagreement on the direction of the shift in the T_m and on the amount of cholesterol necessary to completely abolish the transition. Several recent studies of DPPC–cholesterol systems employing high-sensitivity DSC appear to have resolved this controversy and have revealed additional information about the nature and stoichiometry of cholesterol–phospholipid interactions [100–102]. At cholesterol concentrations between 0–20 mol%, the DSC trace of the main transition can be resolved into two components (see Fig. 5). The sharp component originally centered at the T_m of pure DPPC shifts to slightly lower temperatures as cholesterol is added and its ΔH_{cal} decreases linearly with increasing cholesterol content, falling from an initial value of 8–8.5 kcal/mol to zero at about 20 mol%. The broad component exhibits a slightly higher T_m, which shifts to higher temperatures and is broadened as cholesterol content increases above 20 mol%; the ΔH_{cal} of this broad component initially increases and then decreases with increasing cholesterol content, reaching zero at about 50 mol% cholesterol. A recent dilatometric study has provided support for this analysis [103]. Essentially similar findings have been reported for PE–cholesterol [103] and SM–cholesterol [105] systems, except that in these latter mixtures the T_m of the broad component shifts to lower rather than to higher temperatures. The existence of both a sharp and a broad transition at cholesterol concentrations of <20 mol% appears to be due to a lateral phase separation into cholesterol-free and cholesterol-enriched domains, respectively [100–106]. The reason that cholesterol decreases the T_m of PE and SM while increasing the T_m of PC may be due to the intermolecular hydrogen bonding present in PE and SM bilayers, which may be partially disrupted by the insertion of cholesterol molecules. Incidentally,

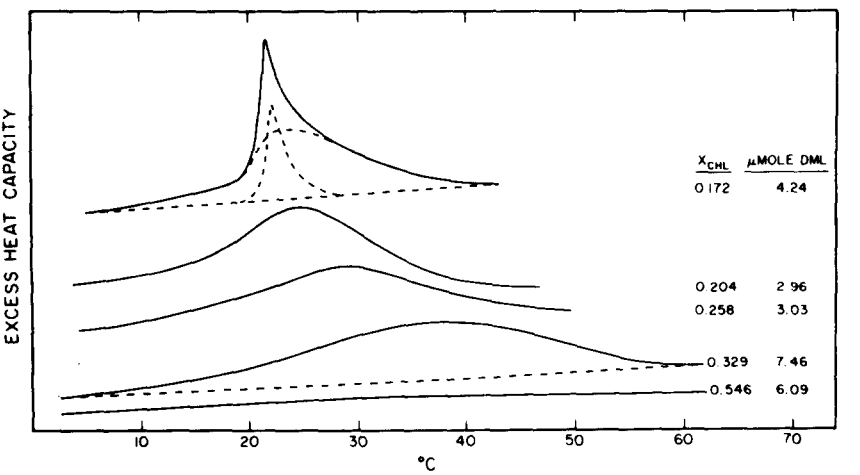

Fig. 5. DSC traces of five mixtures of cholesterol with multilamellar dispersions of DMPC (Reproduced from Mabrey and Sturtevant (1978) [2] with permission from Plenum Press).

the incorporation of small amounts of cholesterol (about 3–7 mol%) into PC or SM dispersions completely abolishes their pretransitions.

Several conventional DSC studies of binary mixtures of two phospholipids exhibiting gel phase immiscibility have indicated that cholesterol may preferentially associate with different lipid classes in such mixtures [58,101,102]. When cholesterol was added to monotectic mixtures of PC–PC, PC–PG, PC–PS or PS–PE, cholesterol preferentially interacted with the lower-melting lipid as indicated by a decrease in ΔH_{cal} and a broadening of the phase transition. In other binary systems, however, cholesterol exhibited a preference for a particular phospholipid whether or not that lipid was the lower or higher-melting component. The order of preference of cholesterol thus established was SM $\gg$ PS $\cong$ PG $>$ PC $\gg$ PE. Cholesterol does not appear, however, to show any calorimetrically detectable preferential affinity in several PC–PE–cholesterol [104] and PC–SM–cholesterol [109] systems which, while non-ideal, were not monotectic. Since the extracted phospholipids from eukaryotic membranes without cholesterol show single relatively broad transitions and no monotectic behavior, it seems unlikely that in biological membranes preferential cholesterol–phospholipid interactions of sufficient strength and specificity occur, inducing the formation of large cholesterol-free and cholesterol-rich domains as apparently occurs in certain non-cocrystallizing binary mixtures of synthetic phospholipids. Cholesterol-poor or cholesterol-rich microdomains or clusters which are also enriched or depleted of certain phospholipid classes may, however, occur.

Effect of small molecules on the thermotropic behavior of phospholipids

A number of lipid-soluble small molecules including drugs like tranquilizers, antidepressants, narcotics and anesthetics, produce biological effects in living cells. Although some of these compounds are known to produce their characteristic effects by interacting with specific membrane proteins, others seem to interact rather non-specifically with the lipid bilayer of many biological membranes. The effect on the gel to liquid-crystalline phase transition profile of synthetic PCs of over one hundred hydrophobic small molecules producing biological effects have now been studied by DSC (for a review see Ref. 110). At least four different types of modified transition profiles can be distinguished. In so-called type C profiles the addition of the additive shifts T_m usually (but not always) to a lower temperature, while having little or no effect on the cooperativity ($\Delta T_{1/2}$) or ΔH_{cal} of the transition; other physical evidence suggests that additives producing this behavior are usually localized in the central region of the bilayer, interacting primarily with the C_9–C_{16} methylene region of the phospholipid hydrocarbon chains. Type A profiles are characterized by a shift in T_m usually to a lower temperature, an increase in $\Delta T_{1/2}$ and a relatively unaffected ΔH_{cal} upon the addition of the appropriate small molecules; these additives appear to be partially buried in the hydrocarbon core of the bilayer interacting primarily with the C_2–C_8 methylene region of the hydrocarbon chains. In type B profiles a shoulder emerges on the main

transition, the area of which increases in conjunction with a corresponding decrease in the area of the original peak as the concentration of additive increases. The total area of both peaks is relatively unchanged, at least at low additive concentration. Additives which produce type B profiles are generally present at the hydrophobic-hydrophilic interface of the bilayer and interact primarily with the glycerol b ckbone of the phospholipid molecules. Finally, type D profiles exhibit a discrete new peak which grows in area at the expense of the parent peak as the additive concentration increases; normally, however, the final ΔH_{cal} and $\Delta T_{1/2}$ values of the new and original peaks are not greatly different. Type D additives usually seem to be located at the bilayer surface and to interact with the phosphorylcholine headgroup. Although this classification is useful not all small molecules produce one of these four types of DSC profiles. Whether or not a consistent relationship exists between the type of transition profile produced by a small molecule and its physiological effects remains to be determined [110].

Free fatty acids occur as minor components of many biological membranes and these compounds can alter the permeability properties of model membranes and the activity of certain enzymes in biomembranes. Moreover, free fatty acids can promote the fusion of phospholipid vesicles and cells. DSC and other physical techniques have been utilized to monitor the effect of different fatty acids on the gel to liquid-crystalline phase transition of synthetic PCs [62,79,111–113]. In general the addition of small amounts of saturated free fatty acids of 12–18 carbons to DPPC multilamellar dispersions results in increased T_m, $\Delta T_{1/2}$ and ΔH_{cal} values for the main transition while abolishing the pretransition. Palmitic acid has the largest effect on DMPC bilayers, probably because the location of the carboxyl group at the hydrophobic-hydrophilic interface results in the palmitic acid hydrocarbon chain having the same effective length as the myristoyl chains in DMPC. Saturated fatty acids with 10 or fewer carbons and unsaturated fatty acids also increase $\Delta T_{1/2}$ but in contrast lower the T_m and ΔH_{cal} values for chain melting. Very long-chain saturated fatty acids (20–22 carbons) increase $\Delta T_{1/2}$ and decrease ΔH_{cal} without affecting T_m, just as does the addition of cholesterol. Interestingly, the presence of saturated or unsaturated fatty acids did not appear to alter lipid fluidity in the liquid-crystalline state as monitored by pyrene eximer fluorescence [113]. The addition of high levels of palmitic acid to DPPC (at a mole ratio of 2 : 1) produces a sharp asymmetric melting profile with a T_m of 61.5°C, only a few degrees below the T_m of DPPE [62]. This observation was taken to support the concept that the lower T_m values characteristic of PCs as compared to PEs may be the result of a destabilizing crowding of the relatively bulky PC head groups.

Lysophospholipids also occur as minor components of most biological membranes and like free fatty acids can affect membrane permeability, promote membrane fusion and modulate the activity of some membrane enzymes. The effect of increasing concentrations of 1-palmitoyl-*sn*-glycerol-3-phosphorylcholine (lysoPPC) on the thermotropic phase behavior of DPPC has been studied by DSC [114]. LysoPPC, which alone exhibited an endothermic transition at 3.4°C, caused

a non-linear decrease in the T_m of DPPC. LysoPPC also caused an initial slight increase in the ΔH_{cal} of DPPC followed by a gradual decrease at higher lysoPPC concentrations, but at low concentrations the $\Delta T_{1/2}$ was unaffected. No phase separation could be detected and the lamellar phase persisted up to 50 mol% lysoPPC. Mixtures of lysoPPC with DOPC or DSPC, or of lysoOPC with either DPPC or DOPC, however, exhibited immiscibility in the PC gel state [115,116]. Small amounts of lysoPPC abolished the pretransition of DPPC. Cholesterol was also found to decrease the ΔH_{cal} of the pure lysoPPC transition, eliminating it at a concentration of about 50 mol%, just as for DPPC. Moreover, cholesterol increased the T_m of the lysoPPC transition [114]. The ether analog of lysoPPC was also found to lower the T_m of DPPE, but in contrast to DPPC the $\Delta T_{1/2}$ was markedly increased as was the ΔH_{cal}, from 8.2 to 18.0 kcal/mol. The very high ΔH_{cal} was ascribed to the breaking of the network of intermolecular hydrogen bonds between the PE molecules caused by the insertion of lysoPPC. The addition of lysoPPC to DPPC–DPPE mixtures, which in the absence of the lysophospholipid exhibited almost complete gel phase miscibility, induced a separation into three different phases. In contrast the addition of the either analog of lysoPPC to DMPE–DSPC mixtures abolished the miscibility gap normally found in this particular binary system [116]. Thus, the effect of lysoPPC on phospholipid mixtures is complex and depends on the nature of both the lysophospholipid and the diacyl phospholipids being studied.

It might be noted that the results above are at variance with those reported by another group using a spectroscopic technique, which supposedly detected lateral phase separations of lysoPPC–DPPC in the gel state [117]. There are a number of instances in the literature of discrepancies between phase diagrams determined by various potentially perturbing spectroscopic methods and by calorimetry (for discussion see Refs. 2, 83). Since, as discussed earlier, the presence of a spectroscopic probe may seriously perturb the system under study and the probe can become localized in non-representative local domains, phase diagrams obtained by spectroscopic means should be accepted only tentatively until confirmed by a more direct non-perturbing technique such as DSC.

Effect of proteins on the thermotropic behavior of phospholipids

Because of their obvious relevance to biological membranes the effect of a number of peptides and proteins on the thermotropic phase behavior of single synthetic phospholipids has been studied by many groups (for a review see Ref. 117). It has been proposed that polypeptides and proteins can be considered as belonging to one of three types according to their characteristic effects on phospholipid gel to liquid-crystalline phase transitions. Type 1 proteins typically produce no change or a modest increase in T_m, a slight increase or no change in $\Delta T_{1/2}$ and an appreciable and progressive increase in ΔH_{cal} as the amount of protein added is increased. These proteins normally do not expand phospholipid monolayers, nor alter the permeability of phospholipid vesicles into which they are incorporated. Type 1

proteins are 'hydrophilic' proteins which are thought to interact with the phospholipid bilayer exclusively by electrostatic forces and as such normally show stronger effects on the phase transitions of charged rather than zwitterionic phospholipids. Examples include poly-L-lysine and ribonuclease. Type 2 proteins produce a decrease in T_m, an increase in $\Delta T_{1/2}$ and a considerable and progressive decrease in ΔH_{cal}; phospholipid monolayers are typically expanded by such proteins and these proteins normally increase the permeability of phospholipid vesicles. These proteins, which are also hydrophilic and include hemoglobin, cytochrome *c* and myelin basic protein, are believed to interact with phospholipid bilayers by a combination of electrostatic and hydrophobic forces, initially adsorbing to the charged polar head groups of the phospholipids and subsequently partially penetrating the hydrophilic-hydrophobic interface of the bilayer to interact with a portion of the hydrocarbon chains. Finally, type 3 proteins usually have little effect on the T_m or $\Delta T_{1/2}$ of the phospholipid phase transition, but ΔH_{cal} decreases linearly with protein concentration. Type 3 proteins are 'hydrophobic' proteins which markedly expand phospholipid monolayers and increase the permeability of phospholipid vesicles. These proteins are thought to penetrate deeply into or through the hydrophobic core of the lipid bilayer, interacting strongly with the phospholipid fatty acyl chains and essentially removing them from participation in the cooperative chain-melting transition. It should be noted, however, that type 3 proteins may also interact electrostatically with phospholipid polar headgroups, particularly with those bearing a net negative charge. For example the hydrophobic integral protein of the myelin membrane, lipophilin, exhibits preferential binding to acidic phospholipids [118] even though it behaves as a type 3 protein calorimetrically and immobilizes and disorders the hydrocarbon chains of its 'boundary lipid' [119]. Similarly glycophorin, a membrane-spanning glycoprotein of the erythrocyte membrane, immobilizes about nine negatively charged phospholipid molecules per molecule of protein via strong electrostatic interactions with the phosphate head group [120]. It should be noted that not all proteins exhibit behavior that can be neatly fitted into the above classification scheme.

A plot of the ΔH_{cal} of a phospholipid gel to liquid-crystalline phase transition versus the protein/phospholipid molar ratio can yield the number of phospholipid molecules withdrawn from the cooperative chain-melting transition by each type 3 protein, when ΔH_{cal} is extrapolated to zero. These values have ranged from 6 and 10 for the small hydrophobic peptides gramicidin [121] and melittin [122], respectively, to about 15 for the relatively small membrane protein lipophilin [123]. For these proteins it appears that only one layer of phospholipid molecules, that is those phospholipids interacting directly with the surface of the protein hydrophobic region, are withdrawn from the cooperative phase transition. On the other hand, the membrane-spanning and somewhat larger bacteriophage M-13 coat protein appears to remove 70–100 phospholipid molecules [124], while the considerably larger hydrophobic protein glycophorin also removes 80–100 molecules from the transition [120]; the membrane-spanning regions of these proteins would

appear to withdraw roughly three phospholipid layers. The vesicular stomatitis virus hydrophobic glycoprotein, whose incorporation into DPPC vesicles decreased the T_m as well as the ΔH_{cal} and also increased $\Delta T_{1/2}$, bound 270 ± 150 phospholipid molecules corresponding to the removal of five to six concentric shells of phospholipid per glycoprotein molecule [125]. The basis for this differing behavior is not presently understood but may be related in part to differential interactions, both electrostatic and hydrophobic, between the non-membrane-spanning regions of these larger proteins and the lipid bilayer.

As previously mentioned, proteins can induce an isothermal lateral phase separation of negatively charged lipids in a somewhat similar manner to Ca^{2+}. A number of intrinsic membrane proteins including lipophilin, several ATPases, and rhodopsin preferentially bind acidic phospholipids in their boundary lipid regions in preference to PC or PE. Also a number of water-soluble proteins such as polylysine, cytochrome *c* and myelin basic protein can act as polycations and separate out acidic lipids even in binary mixtures where these lipids would normally be nearly ideally mixed. Moreover, the relative strengths of the interactions between a type 2 protein such as the myelin basic protein and various negatively charged phospholipids can vary markedly with the nature of the lipid headgroup (for review see Ref. 76). These findings have important implications for biological membranes since the conformation and activity of membrane proteins may be determined by the properties of the lipids in its own micro-environment rather than by the properties of the bulk phase lipids. There is some non-calorimetric evidence that the conformation of several membrane proteins can be altered by varying the nature of the phospholipid head group, the amount of cholesterol present or the fluidity of the lipid bilayer [76].

Studies of biological membranes

Studies of mycoplasma and bacterial plasma membranes

The first biological membrane to be successfully studied by DSC was the plasma membrane of the simple, cell wall-less prokaryote *Acholeplasma laidlawii* B [7]. This mycoplasma possesses a number of advantages for membrane studies including the presence of only a single easily isolated membrane which contains essentially all of the cellular lipid, the absence of a lipid-containing cell wall, the ability to be grown in the presence or absence of cholesterol, and the ability to incorporate substantial quantities of exogenous fatty acids into its membrane glyco- and phospholipids (for a review see Ref. 126). When isolated *A. laidlawii* B membranes or whole cells were analyzed by DSC, two endothermic transitions were observed on the first heating scan. The relatively broad lower-temperature transition was fully reversible, varied in temperature in the expected manner with changes in the chain length and degree of unsaturation of the lipid fatty acyl chains, was broadened and eventually abolished by cholesterol incorporation, and exhibited a total enthalpy

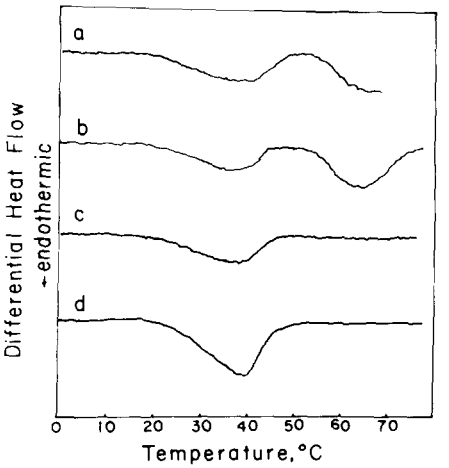

Fig. 6. DSC scans of *A. laidlawii* B (a) whole cells, (b) isolated membranes (first scan), (c) isolated membranes (second scan, after protein denaturation), and (d) aqueous multilamellar dispersion of the total membrane lipids. Cells were grown without fatty acid supplementation at 37°C and were composed largely of saturated fatty acids of 14 and 16 carbon atoms.

change of about 3.6 cal/g, similar to that of mixed-acid synthetic phospholipids Moreover, a very similar transition was observed for protein-free total membrane lipid extracts dispersed in water or aqueous buffer, indicating that this lower-temperature endotherm must originate from the gel to liquid-crystalline phase transition of the membrane lipids of this organism arranged in a bilayer. The second, higher-temperature transition was irreversible, was independent of the membrane lipid fatty acid composition or cholesterol content, was reduced in enthalpy by pronase treatment and was absent in membrane lipid extracts, indicating that it was due to an irreversible thermal denaturation of the membrane proteins. A comparison of the enthalpies for the membranes and extracted lipids revealed that about 90% of the membrane lipid participated in the cooperative chain-melting transition, and the general similarities in the membrane and isolated lipid endotherms suggested that the presence of membrane proteins did not greatly perturb the lipid transition in the intact membrane of the living cell. Subsequent work with high-sensitivity DSC and many other physical techniques have confirmed these findings, not only for *A. laidlawii* B but for a number of bacterial membranes, in particular for unsaturated fatty acid auxotrophs of *Escherichia coli* (see Refs. 1–3 for reviews). The only exception to this general behavior reported so far appears to be the membranes and membrane lipid extracts from the halophilic bacterium *Halobacterium halobium,* where no gel to liquid-crystalline lipid phase transition could be detected by high-sensitivity DSC [127,128]. This is presumably due to the large amounts of the dihydrophytanoyl hydrocarbon chains

present in the membrane lipids of this organism. As discussed earlier, the highly branched synthetic phospholipid diphytanoyl PC does not undergo a discrete cooperative chain-melting transition in the temperature range −120°C to +120°C [35].

It has recently become possible to produce *A. laidlawii* B membranes containing essentially only a single exogenous *n*-saturated, branched chain, unsaturated or cyclopropane fatty acid, by inhibiting de novo fatty acid biosynthesis and chain elongation [129]. Because such membranes lack fatty acid heterogeneity, it is possible to assess the relative contributions of polar head group and fatty acyl group heterogeneity to the relatively broad phase transitions characteristic of native or fatty acid-enriched membranes. It was found that a strong correlation existed between fatty acid heterogeneity and the sharpness of the gel to liquid-crystalline membrane lipid phase transition as detected by DTA, with the transition width decreasing from a normal value of 25–30°C to a limiting value of about 7°C, indicating that fatty acid heterogeneity was the primary contributor to the broad transitions observed in native membranes [130]. Additional DTA studies confirmed that the five major membrane lipids of this organism (two glycolipids, two phospholipids and a phosphorylated glycolipid) show a high degree of miscibility in both the gel and liquid-crystalline states and that Mg^{2+} did not induce a lateral phase separation of the acidic lipid components [131]. Also, the $T_{1/2}$ values of the fatty acid-homogeneous membranes correlated very well with the T_m values of synthetic PCs containing the same acyl chains. Finally, the $T_{1/2}$ of the membrane transitions depended on the nature and chain length of the fatty acyl group present even with comparable levels of enrichment (98–99 mol%).

Studies of the membranes of eukaryotic cells

The presence of high levels of cholesterol in many eukaryotic membranes, particularly plasma membranes, abolishes a discrete cooperative gel to liquid-crystalline membrane lipid phase transition in these systems. Thus, no lipid phase transitions could be detected by DSC or DTA in the cholesterol-rich erythrocyte [132] or myelin [133] membranes. Cholesterol-free lipid extracts of these membranes did, however, exhibit a single broad phase transition, the former centered near 0°C and the latter extending from 25–60°C; the high T_m of the cholesterol-free myelin extract is due primarily to its high content of sphingolipids. Similar reversible thermal transitions, however, were observed by DSC and fluorescence polarization in the microvillus and basolateral regions of rat small intestinal enterocyte plasma membrane and in hydrated lipid extracts [134]. These transitions occurred over a temperature range of about 25–40°C and exhibited very low ΔH_{cal} values of about 0.10–0.15 cal/g for the intact membranes and about 0.40–0.55 cal/g for the extracted lipids. The generally low enthalpies observed were attributed to the large amounts of cholesterol present, and the comparatively lower enthalpy observed in the intact membranes to lipid-protein interactions. The nature of this lipid endotherm remains unclear, although the high levels of cholesterol

and polyunsaturated fatty acyl groups present make it unlikely that it represents a bulk-phase gel to liquid-crystalline transition.

The thermotropic behavior of rat liver microsomal membranes, which contain moderate levels of cholesterol, has been studied by DSC. An early study using conventional DSC revealed a single reversible broad phase transition occurring between −15°C and +5°C in both intact membranes and isolated lipids [135]. A more recent high-sensitivity DSC study confirmed the absence of a reversible phase transition above 0°C [136]. However, rats fed a fat-free diet which increased the degree of saturation of the membrane lipid fatty acids, also exhibited two reversible membrane lipid phase transitions centered at +3°C and +14°C; after protein denaturation, the lower-temperature peak increased in area and shifted to a higher temperature while the higher-temperature lipid peak decreased in area. It thus appears that 'normal' microsomal membrane lipids exist entirely in the fluid state above 0°C, and that the organization of at least a portion of the membrane lipid is dependent on the state of the membrane protein, in contrast to the situation in mycoplasma and bacterial membranes. The existence of a second reversible higher-temperature transition in rat liver microsomal membranes, detected by conventional DSC, has been reported; this transition, which occurred between 18–40°C in intact membranes and between 10–20°C in extracted lipids, was of relatively low enthalpy and was not affected by protein denaturation [137]. It is not clear why this transition could not be detected by high-sensitivity DSC. The molecular basis for this higher-temperature transition, if real, is unknown.

Rat liver mitochondrial membranes which are low in cholesterol, have been studied by several groups using DSC and other techniques. The earliest work with whole mitochondria revealed a reversible broad gel to liquid-crystalline phase transition centered at 0°C in mitochondrial membranes and in extracted lipids [135]. A later study of both intact mitochondria and of isolated inner and outer membranes confirmed these results, except that the outer membrane transition seemed to occur at a slightly lower temperature than did the inner membrane transition [138]. However, a more recent study of the rat liver inner mitochondrial membrane reported a narrower membrane lipid transition centered near +10°C; by artificially increasing cholesterol content some 10-fold to about 30 mol%, the inner membrane gel to liquid-crystalline phase transition could be lowered and broadened, and its ΔH_{cal} reduced to less than 1/10 that of the native membrane [139]. It has also been reported that in beef heart mitochondrial inner membranes a broad reversible endothermic phase transition centered at −10°C occurred; after protein thermal denaturation a new reversible transition of low enthalpy was observed at about 20°C. The extracted lipids exhibited thermal behavior nearly identical to that of the intact protein-denatured membrane, indicating that a latent pool of higher-melting lipids may exist in this membrane [140]. It is clear from all these studies that the lipids of both mitochondrial membranes exist exclusively in the fluid state at physiological temperatures.

One should also mention that DSC has been used to study the individual

protein components of biological membranes of relatively simple protein composition, and the interaction of several of these components with lipids and with other proteins. The red blood cell membrane, which has been most intensively studied, exhibits five discrete protein transitions each of which has been assigned to a specific membrane protein. The response of each of these thermal transitions to variations in temperature and pH as well as to treatment with proteases, phospholipases, specific labeling reagents, and modifiers and inhibitors of selected membrane activities, has provided much useful information on the interactions and functions of these components in the intact erythrocyte membrane [141–144]. Similar approaches have recently been applied to the bovine rod outer segment membrane [145] and to the spinach chloroplast thylakoid membrane [146].

Conclusion

It is clear from theoretical considerations and from the experimental results just summarized that DSC is a very powerful technique for the study of the lipid and protein components of model and biological membranes. With the advent of high-sensitivity instruments capable of analyzing relatively dilute samples using slow scan speeds, the application of this non-perturbing technique to problems in lipid and membrane research should increase even further in future years.

Acknowledgements

Work from the author's laboratory was supported by Operating and Major Equipment Grants from the Medical Research Council of Canada.

References

1 D.L. Melchior and J.M. Steim, Annu. Rev. Biophys. Bioeng., 5 (1976) 205.
2 S. Mabrey and J.M. Sturtevant, in: E.D. Korn (Ed.), Methods in Membrane Biology, Vol. 9, Plenum Press, New York, 1978, p. 237.
3 D.L. Melchior and J.M. Steim, in: D.A. Cadenhead and J.F. Danielli (Eds.), Progress in Surface and Membrane Science, Vol. 13, Academic Press, New York, 1979, p. 211.
4 D. Chapman, R.M. Williams and B.D. Ladbrooke, Chem. Phys. Lipids, 1 (1967) 445.
5 F. Reiss-Husson and V. Luzzati, Adv. Med. Phys., 11 (1967) 87.
6 B.D. Ladbrooke and D. Chapman, Chem. Phys. Lipids, 3 (1969) 304.
7 J.M. Steim, M.E. Tourtellotte, J.C. Reinert, R.N. McElhaney and R.L. Rader, Proc. Natl. Acad. Sci. U.S.A., 63 (1969) 104.
8 V. Luzzati, in: D. Chapman (Ed.), Biological Membranes, Vol. 1, Academic Press, New York, 1968, p. 71.
9 D.M. Engelman, J. Mol. Biol., 58 (1971) 153.
10 G.G. Shipley, in: D. Chapman and D.F.H. Wallach (Eds.), Biological Membranes, Vol. 2, Academic Press, New York, 1973, p. 1.

11 Y.K. Levine, Prog. Biophys. Mol. Biol., 24 (1972) 1.
12 D. Chapman, in: H. Eisenberg, E. Katchalski-Katzir and L.A. Manson (Eds.), Biomembranes, Vol. 7, Plenum Press, New York, 1975, p. 1.
13 J.F. Nagle and D.A. Wilkinson, Biophys. J., 23 (1978) 159.
14 D.A. Wilkinson and J.F. Nagle, Biochemistry, 20 (1981) 187.
15 N. Albon and J.M. Sturtevant, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 2258.
16 R.N. McElhaney, J. Mol. Biol., 84 (1974) 145.
17 A.G. Lee, Prog. Biophys. Mol. Biol., 29 (1975) 3.
18 J.E. Cronan and E.P. Gelman, Bacteriol. Rev., 39 (1975) 232.
19 R.N. McElhaney, in: S. Razin and S. Rottem (Eds.), Current Topics in Membranes and Transport, Vol. 15, Academic Press, New York, in press.
20 B.R. Lentz, E. Freire and R.L. Biltonen, Biochemistry, 17 (1978) 4475.
21 K.C. Cho, C.L. Choy and K. Young, Biochim. Biophys. Acta, 663 (1981) 14.
22 R.N. McElhaney and K.A. Souza, Biochim. Biophys. Acta, 443 (1976) 348.
23 D.A. Cadenhead, B.M.J. Kellner, K. Jacobson and D. Papahadjopoulos, Biochemistry, 16 (1977) 5386.
24 F. Podo and J.K. Blasie, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 1032.
25 S. Schreier, C.F. Polnaszek and I.C.P. Smith, Biochim. Biophys. Acta, 515 (1978) 375.
26 B. Mely-Goubert and M.H. Freedman, Biochim. Biophys. Acta, 601 (1980) 315.
27 M.G. Taylor and I.C.P. Smith, Biochim. Biophys. Acta, 599 (1980) 140.
28 J.R. Silvius, B.D. Read and R.N. McElhaney, Biochim. Biophys. Acta, 555 (1979) 175.
29 J.R. Silvius and R.N. McElhaney, Chem. Phys. Lipids, 24 (1979) 287.
30 J.R. Silvius and R.N. McElhaney, Chem. Phys. Lipids, 25 (1979) 125.
31 J.R. Silvius and R.N. McElhaney, Chem. Phys. Lipids, 26 (1980) 67.
32 J.T. McGarrity and J.B. Armstrong, Biochim. Biophys. Acta, 640 (1981) 544.
33 S. Legendre, L. Letellier and E. Shechter, Biochim. Biophys. Acta, 602 (1980) 491.
34 H. Lindsey, N.O. Petersen and S.I. Chan, Biochim. Biophys. Acta, 555 (1979) 147.
35 K.M.W. Keough and P.J. Davis, Biochemistry, 18 (1979) 1453.
36 S.C. Chen and J.M. Sturtevant, Biochemistry, 20 (1981) 713.
37 J. Stumpel, A. Nicksch and H. Eibl, Biochemistry, 20 (1981) 662.
38 A. Seelig and J. Seelig, Biochim. Biophys. Acta, 406 (1975) 1.
39 R.A. Haberkorn, R.G. Griffin, M.D. Meadows and E. Oldfield, J. Am. Chem. Soc., 99 (1977) 7353.
40 E. Oldfield, M. Meadows, D. Rice and R. Jacobs, Biochemistry, 17 (1978) 2727.
41 G. Buldt, H.U. Gally, A. Seelig, J. Seelig and G. Zaccai, Nature (London), 271 (1978) 182.
42 P.B. Hitchcock, R. Mason, K.M. Thomas and G.G. Shipley, Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 3036.
43 R.H. Pearson and I. Pascher, Nature (London), 281 (1979) 499.
44 B. de Kruyff, R.A. Demel and L.L.M. van Deenen, Biochim. Biophys. Acta, 255 (1972) 331.
45 B. de Kruyff, R.A. Demel, A.J. Slotboom, L.L.M. van Deenen and A.F. Rosenthal, Biochim. Biophys. Acta, 307 (1973) 1.
46 J.A.F. Op den Kamp, M.T. Kauerz and L.L.M. van Deenen, Biochim. Biophys. Acta, 406 (1975) 169.
47 P.J. Davis, K.P. Coolbear and K.M.W. Keough, Can J. Biochem., 38 (1980) 851.
48 M.R. Bunow and B. Bunow, Biophys. J., 27 (1979) 325.
49 J.R. Silvius, in: P.C. Jost and O.H. Griffith (Eds.), Protein-Lipid Interactions. Academic Press, New York, in press.
50 J. Seelig, R. Dijkman and G.H. de Haas, Biochemistry, 19 (1980) 2215.
51 P.G. Barton and F.D. Gunstone, J. Biol. Chem., 250 (1975) 4470.
52 D. Chapman, J. Urbina and K.M. Keough, J. Biol. Chem., 249 (1974) 2512.

53 S.A. Simon, L.J. Lis, J.W. Kauffman and R.C. McDonald, Biochim. Biophys. Acta, 375 (1975) 317.
54 D. Chapman, W.E. Peel, B. Kingston and T.H. Lilley, Biochim. Biophys. Acta, 464 (1977) 260.
55 W.F. Graddick, J.B. Stamatoff, P. Eisenberger, D.W. Berreman and N. Spielberg, Biochem. Biophys. Res. Commun., 88 (1979) 907.
56 D.J. Vaughan and K.M. Keough, FEBS Lett., 47 (1974) 158.
57 S. Mabrey and J.M. Sturtevant, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 3862.
58 P.W.M. van Dijck, B. de Kruijff, L.L.M. van Deenen, J. de Gier and R.A. Demel, Biochim. Biophys. Acta, 455 (1976) 576.
59 M.B. Jackson and J.M. Sturtevant, J. Biol. Chem., 252 (1977) 4749.
60 R.D. Yang, K.M. Patel, H.J. Pownall, R.D. Knapp, L.A. Sklar, R.B. Crawford and J.D. Morrisett, J. Biol. Chem., 254 (1979) 8256.
61 D.A. Wilkinson and J.F. Nagle, Biochemistry, 20 (1981) 187.
62 S. Mabrey and J.M. Sturtevant, Biochim. Biophys. Acta, 486 (1977) 444.
63 J.F. Nagle, J. Membr. Biol., 27 (1976) 233.
64 H. Eibl, in: W.H. Kuman and R.T. Holman (Eds.), Polyunsaturated Fatty Acids. American Oil Chemists Society, 1977, p. 229.
65 P.R. Cullis and B. de Kruijff, Biochim. Biophys. Acta, 513 (1978) 31.
66 K. Jacobson and D. Papahadjopoulos, Biochemistry, 14 (1975) 152.
67 P.W.M. van Dijck, P.H.J.T. Ververgaert, A.J. Verkleij, L.L.M. Deenen and J. de Gier, Biochim. Biophys. Acta, 406 (1975) 465.
68 P.W.M. van Dijck, B. de Kruijff, A.J. Verkleij, L.L.M. van Deenen and J. de Gier, Biochim. Biophys. Acta, 512 (1978) 84.
69 E.J. Findlay and P.G. Barton, Biochemistry, 17 (1978) 2400.
70 M.-M. Sacre, W. Hoffman, M. Turner, J.-F. Tocanne and D. Chapman, Chem. Phys. Lipids, 25 (1979) 69.
71 S. Rainier, M.K. Jain, F. Ramirez, P.V. Ioannou, J.F. Marecek and R. Wagner, Biochim. Biophys. Acta, 558 (1979) 187.
72 R.C. MacDonald, S.A. Simon and E. Baer, Biochemistry, 15 (1976) 885.
73 H. Eibl and A. Blume, Biochim. Biophys. Acta, 553 (1979) 476.
74 K. Harlos, J. Stumple and H. Eibl, Biochim. Biophys. Acta, 555 (1979) 409.
75 A. Blume and H. Eibl, Biochim. Biophys. Acta, 558 (1979) 13.
76 J.M. Boggs, Can. J. Biochem., 58 (1980) 755.
77 B. de Kruijff, P.R. Cullis and G.K. Radda, Biochim. Biophys. Acta, 406 (1975) 6.
78 J. Suurkuusk, B.R. Lentz, Y. Barenholz, R.L. Biltonen and T.E. Thompson, Biochemistry, 15 (1976) 1393.
79 H.L. Kantor, S. Mabrey, J.H. Prestegard and J.M. Sturtevant, Biochim. Biophys. Acta, 466 (1977) 402.
80 P.W.M. van Dijck, B. de Kruijff, P.A.M.M. Aarts, A.J. Verkleij and J. de Gier, Biochim. Biophys. Acta, 506 (1978) 183.
81 B. Gruenwald, S. Stankowski and A. Blume, FEBS Lett., 102 (1979) 227.
82 M.C. Phillips, B.D. Ladbrooke and D. Chapman, Biochim. Biophys. Acta, 193 (1970) 35.
83 P.W.M. van Dijck, A.J. Kaper, H.A.J. Oonk and J. de Gier, Biochim. Biophys. Acta, 470 (1977) 58.
84 A. Blume and T. Ackermann, FEBS Lett., 43 (1974) 71.
85 A.G. Lee, Biochim. Biophys. Acta, 472 (1977) 285.
86 T.P. Stewart, S.W. Hui, A.R. Portis and D. Papahadjopoulos, Biochim. Biophys. Acta, 556 (1979) 1.
87 A.W. Clowes, R.J. Cherry and D. Chapman, Biochim. Biophys. Acta, 249 (1971) 301.
88 L.O. Sillerud, D.E. Schafer, R.K. Yu and W.H. Konigsberg, J. Biol. Chem., 254 (1979) 10876.

89 Y. Barenholz and T.E. Thompson, Biochim. Biophys. Acta, 604 (1980) 129.
90 A.G. Lee, Biochim. Biophys. Acta, 507 (1978) 433.
91 P.H. Von Dreele, Biochemistry, 17 (1978) 3939.
92 S. Ohnishi and T. Ito, Biochem. Biophys. Res. Commun., 51 (1973) 132.
93 D. Papahadjopoulos, G. Poste, B.E. Schaeffer and W.J. Vail, Biochim. Biophys. Acta, 352 (1974) 10.
94 A.G. Lee, Biochim. Biophys. Acta, 413 (1975) 11.
95 H.-J. Galla and E. Sackman, Biochim. Biophys. Acta, 401 (1975) 509.
96 H.-J. Galla and E. Sackman, J. Am. Chem. Soc., 97 (1975) 4114.
97 T. Ito and S.-I. Ohnishi, Biochim. Biophys. Acta, 352 (1974) 29.
98 S.I. Ohnishi and T. Ito, Biochemistry, 13 (1974) 881.
99 R.A. Demel and B. de Kruyff, Biochim. Biophys. Acta, 457 (1976) 109.
100 J.M. Sturtevant, in: B. Kursunoglu, S. Mintz and S. Widmayer (Eds.), Quantum Statistical Mechanics in the Natural Sciences. Plenum Press, New York 1974, p. 63.
101 T.N. Estep, D.B. Mountcastle, R.L. Biltonen and T.E. Thompson, Biochemistry, 17 (1978) 1984.
102 S. Mabrey, P.L. Mateo and J.M. Sturtevant, Biochemistry, 17 (1978) 2464.
103 D.L. Melchior, F.J. Scavitto and J.M. Steim, Biochemistry, 19 (1980) 4828.
104 A. Blume, Biochemistry, 19 (1980) 4908.
105 T.N. Estep, D.B. Mountcastle, Y. Barenholz, R.L. Biltonen and T.E. Thompson, Biochemistry, 18 (1979) 2112.
106 B. Snyder and E. Freire, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 4055.
107 R.A. Demel, J.W.C.M. Jansen, P.W.M. van Dijck and L.L.M. van Deenen, Biochim. Biophys. Acta, 465 (1977) 1.
108 P.W.M. van Dijck, Biochim. Biophys. Acta, 555 (1979) 89.
109 W.I. Calhoun and G.G. Shipley, Biochemistry, 18 (1979) 1717.
110 M.K. Jain and N.M. Wu, J. Membr. Biol., 34 (1977) 157.
111 A.W. Eliasz, D. Chapman and D.F. Ewing, Biochim. Biophys. Acta, 448 (1976) 220.
112 J.M.H. Kremer and P.H. Wiersema, Biochim. Biophys. Acta, 471 (1977) 348.
113 J.R. Usher, R.M. Epand and D. Papahadjopoulos, Chem. Phys. Lipids, 22 (1978) 245.
114 W.E. Klopfenstein, B. de Kruijff, A.J. Verkleij, R.A. Demel and L.L.M. van Deenen, Chem. Phys. Lipids, 13 (1974) 215.
115 C.J.A. van Echteld, B. de Kruijff and J. de Gier, Biochim. Biophys. Acta, 595 (1980) 71.
116 A. Blume, B. Arnold and H.U. Weltzien, FEBS Lett., 61 (1976) 199.
117 D. Papahadjopoulos, J. Colloid Interface Sci., 58 (1977) 459.
118 J.M. Boggs, D.D. Wood, M.A. Moscarello and D. Papahadjopoulos, Biochemistry, 16 (1977) 2325.
119 J.M. Boggs, W.J. Vail and M.A. Moscarello, Biochim. Biophys. Acta, 448 (1976) 517.
120 E.J.J. van Zoelen, P.W.M. van Dijck, B. de Kruijff, A.J. Verkleij and L.L.M. van Deenen, Biochim. Biophys. Acta, 514 (1978) 9.
121 D. Chapman, B.A. Cornell, A.W. Eliasz and A. Perry, J. Mol. Biol., 113 (1977) 517.
122 C. Mollay, FEBS Lett., 64 (1976) 65.
123 D. Papahadjopoulos, W.J. Vail and M. Moscarello, J. Membr. Biol., 22 (1975) 143.
124 D. Kimelman, E.S. Tecoma, P.K. Wolber, B.S. Hudson, W.T. Wickner and R.D. Simoni, Biochemistry, 18 (1979) 5874.
125 W.A. Petri, T.N. Estep, R. Pal, T.E. Thompson, R.L. Biltonen and R.R. Wagner, Biochemistry, 19 (1980) 3088.
126 S. Razin, in: D.A. Cadenhead, J.F. Danielli and M.D. Rosenberg (Eds.), Progress in Surface and Membrane Science, Vol. 9, Academic Press, New York, 1975, p. 257.
127 M.B. Jackson and J.M. Sturtevant, Biochemistry, 17 (1978) 911.
128 M.B. Jackson and J.M. Sturtevant, Biochemistry, 17 (1978) 4470.

129 J.R. Silvius and R.N. McElhaney, Can. J. Biochem., 56 (1978) 462.
130 J.R. Silvius and R.N. McElhaney, Nature (London), 272 (1978) 645.
131 J.R. Silvius, N. Mak and R.N. McElhaney, Biochim. Biophys. Acta, 597 (1980) 199.
132 B.D. Ladbrooke, R.M. Williams and D. Chapman, Biochim. Biophys. Acta, 150 (1968) 333.
133 B.D. Ladbrooke, T.J. Jenkinson, V.B. Kamat and D. Chapman, Biochim. Biophys. Acta, 164 (1968) 101.
134 T.A. Brasitus, A.R. Tall and D. Schachter, Biochemistry, 19 (1980) 1256.
135 J.F. Blazyk and J.M. Steim, Biochim. Biophys. Acta, 266 (1972) 737.
136 S. Mabrey, G. Powis, J.B. Schenkman and T.R. Tritton, J. Biol. Chem., 252 (1977) 2929.
137 D. Bach, I. Bursuker and R. Goldman, Biochim. Biophys. Acta, 469 (1977) 171.
138 C.R. Hackenbrock, M. Hochli and R.M. Chau, Biochim. Biophys. Acta, 455 (1976) 466.
139 T.D. Madden, C. Vigo, K.R. Bruckdorfer and D. Chapman, Biochim. Biophys. Acta, 599 (1980) 528.
140 J.F. Blazyk and J.L. Newman, Biochim. Biophys. Acta, 600 (1980) 1007.
141 J.F. Brandts, L. Erickson, K. Lysko, A.T. Schwartz and R.D. Taverna, Biochemistry, 16 (1977) 3450.
142 J.F. Brandts, R.D. Taverna, E. Sadasivan and K.A. Lysko, Biochim. Biophys. Acta, 512 (1978) 566.
143 J.W. Snow, J.F. Brandts and P.S. Low, Biochim. Biophys. Acta, 512 (1978) 579.
144 J.W. Snow, J. Vincentelli and J.F. Brandts, Biochim. Biophys. Acta, 642 (1978) 418.
145 G. Miljanich, M.F. Brown, E.A. Dratz, S. Mabrey and J.M. Sturtevant, Biophys. J., 16 (1976) 37a.
146 W.A. Cramer, J. Whitmarsh and P.S. Low, Biochemistry, 20 (1981) 157.