

Differential Scanning Calorimetry of Bilayer Membrane Phase Transitions^{1,2}

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Background Reading: D. L. Nelson, M. M. Cox, Lehninger: Principles of Biochemistry, 3rd Ed., Worth, New York, NY, 2000. pp 369-71, 391-395.

Introduction:

The goal of this experiment is to use Differential Scanning Calorimetry (DSC) to study the gel to liquid-crystalline phase transitions of phospholipid bilayers. Phospholipids are amphipathic molecules, meaning they have both polar and nonpolar segments. Phospholipids have hydrophilic headgroups and hydrophobic tails. They spontaneously assemble into bilayer structures in aqueous solutions in which the headgroups are on the surfaces exposed to water and the hydrophilic tails are directed inwards, Figure 1.

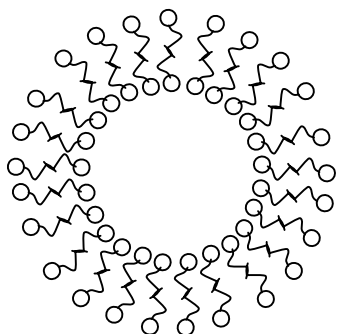


Figure 1. Liposome formed from a phospholipid bilayer.

The assembly is entropy driven due to the release of water molecules from the solvation shell of the phospholipids as the lipids aggregate into the bilayer. Liposomes are produced when the phospholipid bilayer forms around an aqueous cavity. Liposomes are a good model system for the cell membrane. Cell membranes, however, also contain embedded proteins and other lipids, such as cholesterol. Liposomes are useful in their own right, as aids in pharmaceutical delivery and in cosmetic preparations. The liposomes prepared in this experiment form as multilayer vesicles or MLVs.³ MLVs are like an onion with concentric spheres of phospholipid bilayers with diameters between 1000 and 50,000 Å.

In the liposomes, in the lower temperature gel state, the phospholipids are tightly packed by strong van der Waals forces. At the onset of the phase transition, the phospholipids “cooperatively” melt.³ In the resulting liquid-crystalline state, the phospholipids are more loosely associated, owing to weakened van der Waals forces between the acyl chains, weakened polar interactions of the phospholipid headgroups, and a lateral expansion of the acyl chains. Because these weak van der Waals interactions dictate the structure of the membrane, the acyl chain length and the identity of the phospholipid headgroup are the major contributors to changes in the nature of the gel to liquid-crystalline phase transition.^{3,4}

The predominant phospholipids in most membranes are phosphoacylglycerols, phosphate esters of glycerol. Phospholipids have three main functional groups: 1) two long acyl chains, usually with an even number of carbon atoms; 2) the glycerol component; and 3) the phosphate headgroup. Figure 2 shows the structure of the class of phospholipids to be studied in this experiment, phosphatidylcholines (lecithins).

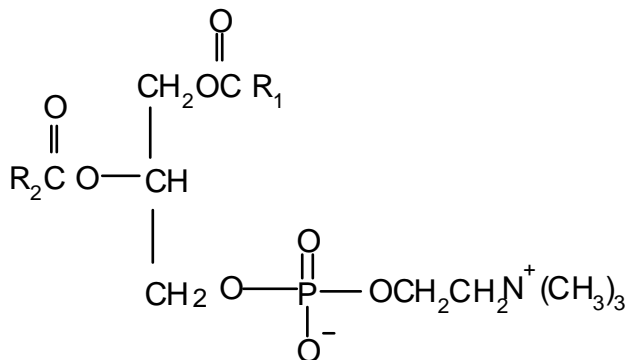


Figure 2: Phosphatidylcholine. R_1 and R_2 are saturated acyl chains derived from fatty acids. R_1 and R_2 are the same in our experiments.

These molecules are named according to R_1 and R_2 , the long-chain hydrocarbon tails of the fatty acids, and the segment attached to the phosphate. Choline is attached to the phosphate for the series of lipids in this laboratory. Choline is indicated as the "C" in "PC." We will examine the following phospholipids.

- Dimyristoyl phosphatidylcholine (DMPC, 14:0 PC, dimyristoyl lecithin)
- Dipalmitoyl phosphatidylcholine (DPPC, 16:0 PC, dipalmitoyl lecithin)
- Distearoyl phosphatidylcholine (DSPC, 18:0 PC, distearoyl lecithin)

The numbers in the abbreviated version of the name represent the number of carbons in the acyl chain (e.g. 14) and the number of double bonds in the same chain (e.g. 0).

We will study the effect of phospholipid chain length on the gel to liquid-crystal phase transition.

Cooperative Phase Transitions:

The gel to liquid-crystalline phase transition is highly "cooperative." In cooperative transitions, well before the transition temperature is reached from below, the molecules begin to reorganize and to move in consort with each other. In other words, the molecules cooperate with each other in gaining new motional freedom; when one molecule picks up motional energy then other nearby molecules find it easier to add motional energy. As the temperature approaches the transition temperature, T_m , the distance range of this cooperation increases. Near the phase transition temperature, you can picture islands of lipids in a more mobile phase intermixed with the less mobile gel phase. The number of molecules on average in these disordered "islands" is called the cooperative unit, C.U. In these islands the motions of the molecules are highly correlated. These correlated interactions aid in the sudden change of order at the phase transition temperature. The larger the cooperative unit, the narrower the phase transition temperature range.

The gel to liquid-crystalline phase transition is first-order with some of the characteristics of second-order transitions. First-order phase transitions have a change in enthalpy and volume at the phase transition temperature. In other words, in first-order transitions there is an abrupt change in the properties of the system at the phase transition temperature. In this respect, a first-order phase transition is "completely correlated," that is completely cooperative. In a first-order phase transition all the molecules undergo the phase transition together, subject only to the availability of thermal energy. A pure first-order transition has an infinitely sharp transition. Second-order transitions do not have enthalpy and volume changes at the transition temperature. For second order phase transitions, the formation of cooperative, correlated motions with limited range broadens the transition by pre-transition effects (i.e. the domains anticipate the transition or "start the transition" early). In the DSC of synthetic phospholipids the limited cooperativity of the transition results in a small peak at a lower temperature than the main melting peak, called the pre-transition, as well as a broadening of the main melting transition.³⁻⁵

Differential Scanning Calorimetry:

The DSC contains a sample cell and a reference cell that are maintained at the same temperature. As an experiment proceeds, the sample and reference cells are raised in temperature in a controlled manner such that the two cells always are maintained at the same temperature. The power supplied to heat each cell is monitored during this process. When a phase transition occurs in the sample cell, there is a difference in the power needed to heat the two cells. The power required to maintain both cells at the same temperature is measured and converted to give an output of heat capacity versus temperature. The heat capacity versus temperature curve is analyzed to determine the transition temperature, T_m , and the calorimetric enthalpy of transition, ΔH_{cal} .

Theory:

For first order phase transitions such as the bilayer gel to liquid-crystalline transition, the transition temperature, T_m , is where the heat capacity, C_p , reaches its maximum value. The value of the calorimetric enthalpy (ΔH_{cal}) for the phase transition is determined by integrating the area under the peak.

$$\Delta H_{cal} = \int C_p dT \quad (1)$$

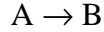
From these values, the entropy of the phase transition is determined:

$$\Delta S = \frac{\Delta H_{cal}}{T_m} \quad (2)$$

Comparison of ΔH_{cal} , ΔS and T_m shows the effect of a structural modification (e.g. chain length) on the thermodynamics of the phase transition. However, unlike a simple organic compound's crystal to liquid melting transition, the phase transition in bilayers involves more than just the initial and final states. In fact, intermediate "states" are formed during the transition, and a "non-two-state" model is necessary for phospholipids in liposomes.⁶⁻⁸ These intermediate states result from the formation of domains (e.g. disordered, mobile areas within the gel phase) before the

phase transition temperature, and are due to lateral movement of the phospholipids within the bilayer. The asymmetric shape of the DSC peak reflects the fact that a non-two-state transition is occurring.

In order to fit adequately these data, a “non-two-state” model is required. For any phase transition that occurs between two phases, A and B,



an equilibrium constant characterizes this process:

$$K = \frac{a_B}{a_A} \quad (3)$$

where a_B and a_A represent the activities of each phase. These activities are determined by the domain sizes of the two phases (see the discussion of cooperative transitions, above). The temperature dependence of the equilibrium constant is related to the enthalpy by the van't Hoff equation (4):

$$\left(\frac{\partial \ln K}{\partial T} \right)_p = \frac{\Delta H_{vH}}{RT^2} \quad (4)$$

The van't Hoff enthalpy, ΔH_{vH} , is equal to the amount of heat required for each cooperative unit to undergo the phase transition. The units are energy/cooperative unit. For a first-order two-state transition, the van't Hoff enthalpy is equal to the calorimetric enthalpy, ΔH_{cal} . In other words, the heat effect for the transition $A \rightarrow B$ is the calorimetric enthalpy, which correspondingly governs the distribution between the two phases. If $\Delta H_{vH} < \Delta H_{cal}$ the process involves one or several intermediate stages, such as $A \rightarrow B \rightarrow C$ and is called non-two state. If $\Delta H_{vH} > \Delta H_{cal}$ the process involves cooperativity, but is not "completely cooperative" as in a first order transition. In other words, the distribution of molecules between the two phases is much more temperature dependent than the actual heat effect of the phase transition due to cooperative motion of the molecules. Therefore, for a non-two-state transition or a partially cooperative transition there are two separate enthalpy parameters, ΔH_{vH} and ΔH_{cal} . After subtracting a baseline from the data, which negates any temperature dependence of ΔH_{cal} , we use equation (4) to obtain an expression to fit our data:^{7,8}

$$C_p(T) = \frac{K(T) \Delta H_{vH} \Delta H_{cal}}{(1 + K(T))^2 RT^2} \quad (5)$$

where $K(T)$ is just the equilibrium constant (3), which is obtained as a function of temperature by solving (4) for $K(T)$:

$$K(T) = e^{-\frac{\Delta H_{vH}}{RT} \left(1 - \frac{T}{T_m} \right)} \quad (6)$$

The software provided by Microcal, Origin, completes this fit and provides the values of ΔH_{cal} , ΔH_{vH} and T_m .

For a more physical picture of the van't Hoff enthalpy, we note that ΔH_{vH} can be calculated directly from the calorimetric data. First, the C_p vs. T output scan from the calorimeter is

integrated to form a plot of the enthalpy for the phase transition, ΔH_{cal} . The maximum of the C_p vs. T curve is $C_{p \text{ max}}$. The van't Hoff enthalpy for the equilibrium is given by:⁸

$$\Delta H_{\text{vH}} = 4 RT_m^2 \frac{C_{p \text{ max}}}{\Delta H_{\text{cal}}} \quad (7)$$

A sharper transition results in a larger value of ΔH_{vH} , since $C_{p \text{ max}}$ is larger. The sharpness of the transition can also be characterized by the full width at half-maximum, FWHM, of the C_p vs. T peak, $\Delta T_{1/2}$. Sharp transitions have a large ΔH_{vH} , and correspondingly small $\Delta T_{1/2}$.

Because the units of ΔH_{vH} are energy/cooperative unit, and ΔH_{cal} is energy/mole, the ratio of the two ($\Delta H_{\text{vH}}/\Delta H_{\text{cal}}$) gives the value of moles (or molecules) per cooperative unit:

$$C.U. = \frac{\Delta H_{\text{vH}}}{\Delta H_{\text{cal}}} \quad (8)$$

The larger the value of C.U., the more cooperative the phase transition. Therefore, cooperative phase transitions have larger ΔH_{vH} . The value of $\Delta T_{1/2}$ can be used as a qualitative measure of molecular cooperativity. Wider peaks correspond to less cooperative phase transitions.

The concept of molecular cooperativity is used for proteins to determine the number of subunits involved in a transition. The use of this concept for phospholipid bilayers is controversial, but the value of C.U. or $\Delta T_{1/2}$ can give a relative measure of the cooperativity of the bilayer phase transition.

Experimental:

Note: Chloroform is a suspected carcinogen. Use gloves when handling chloroform solutions.

Sample preparation:

Degassed water: Place a glass vial containing 3 mL of reagent grade water in the degassing apparatus adjacent to the calorimeter. Add a stirring bar, put on the lid, and set the timer to start the vacuum pump. Adjust the stirring so that it is vigorous, but not turbulent. Allow the sample to degas for five minutes.

Phospholipid bilayers:

Previously prepared stock solutions (2.0 mg/mL) of DMPC (C14:0 PC), DPPC (C16:0 PC), and DSPC (C18:0 PC) in chloroform will be provided. Transfer 2.0 mL of a stock solution with a glass pipette or graduated cylinder to a 25-mL pear-shaped flask. Place the flask on a rotary evaporator in a hot water bath (~70°C) and evaporate to dryness. Don't use any grease on the joints. It may be necessary to place the flask very briefly on a vacuum line to assure complete dryness. Because the rotary evaporator removed the solvent while spinning the flask, the lipids should now be uniformly distributed on the walls of the flask. Add 2.0 mL of degassed distilled water and two small glass beads to the flask using a glass pipette or graduated cylinder. Swirl the flask in a hot water bath above the expected phase transition temperature until a cloudy dispersion forms and the walls of the flask appear clean. The glass beads aid in removing the phospholipids from the walls of the pear-shaped flask and then mixing them in the aqueous

suspension. The cloudy dispersion is due to the presence of large multi-lamellar vesicles (on the order of 500 – 10,000 Å) scattering the light coming into the solution. It is necessary to heat the flask above the phase transition, so that when it cools an ordered gel phase can be formed in the MLVs. The phospholipid bilayer is now available for analysis in the calorimeter as an aqueous dispersion.

Instrument Start up:

The DSC is controlled through the DSC VPViewer software. Normally this program should be running and the instrument turned on and equilibrating when you start. If it is not, double click on the DSC VPViewer icon and allow the calorimeter to warm up. The password for the account is "microcal". Pull down the DSC menu, slide right on DSC Scan Mode, and chose Conventional DSC.

Sample loading:

Before loading samples, use the needle guide and the glass, long-needle syringe to remove the contents of the sample and reference cells. The needle guide is the plastic funnel with the metal bar across the top. Use the same syringe to add degassed water (~0.75 mL) and phospholipid samples (~0.75 mL) to the reference and sample chambers respectively. Use the needle guide to help you. Fill the cells slowly. You should add liquid until you see the liquid begin to fill the cavity around the base of the syringe. When the liquid has been added, use 2-3 short small bursts from the syringe and then gently withdraw the syringe. Remove the needle guide. Withdraw excess liquid using the level adjusting syringe, thus making sure that the cells are filled to exactly the same level. After the cells are full, put on the cap by first tightening the metal ring, and then quickly tightening the white cap. Make the white cap firm, but do not overtighten.

Experimental Parameters:

The Cell 1 conventional DSC Run Parameters window should be displayed in which you will enter the run parameters for your experiment. Enter a filename and parameters appropriate for your experiment. You should do one scan. Scan from at least 20°C below and to 20°C above the expected transition temperature. For DMPC use a temperature scan range of 5-45°C, for DPPC 20-60°C, and for DSPC 35-70°C. Set the PreScan Thermostat time to 10 minutes. Set the scan rate to 60 degrees/hour and a filter setting of 4 sec. Set the PostCycle Thermostat to 20° (unless you are planning to run DMPC next, for which 5°C should be used).

Click the Start button. The DSC will automatically equilibrate and then start the scan. While a scan is in progress you can prepare your next sample.

Analysis of data from the DSC is performed by MicroCal Origin, a general purpose data analysis and plotting software program. This software is available on the computer that runs the DSC.

Calculations:

Calculate the molar concentration of your sample. (You might try the www.avantilipids.com Web site for the molar masses.)

Origin: For your experiments, determine values for ΔH_{cal} , ΔH_{vH} , T_m , and C.U. using the “Non-2-State: Cursor Init” model to fit the data. The designation as a "Non-2-State" transition is a bit

of a misnomer, but the same analysis is used for non-two state and partially cooperative type transitions. Also measure an approximate value for $\Delta T_{1/2}$ (the width of your peak at 1/2 the maximum height). The instructions below will take you through this process step-by-step.

1. On the desk-top, double click on the "Microcal, LLC,DSC" icon.
2. In the new window, click on the "Read Data" button. In the file-librarian dialog box select your data file. Click on the "AddFiles(s)" button. Click on OK.
3. Click on the Normalize Concentration button. Input the molar concentration. The cell volume should already be entered properly (0.5129 mL).
4. Pull down the Peak menu and choose Start Baseline Session...
5. In the Baseline session pull down the Adjust menu and select Move Segments by Cursor. In the thermogram window, drag the line segment endpoints to delineate the flat baseline portions just before and after the main melting peak. (You can go under the pre-transition peak if necessary.)
6. Pull down the Baseline menu and choose Progress Baseline. Inspect the proposed baseline to make sure it matches the flat portions of the baseline. If not, return to step 5.
7. Click on the OK menu entry in the Baseline sessions menu bar. Click on Yes in the pop-up dialog. The base line should be subtracted and the thermogram redisplayed. The sections you chose should be plotted at 0.0 kcal/mol/°C.
8. Pull down the DSC menu and choose "Non-2-state: Cursor Init." Accept the next dialog with 1 fitted peak. Then double click on the top of the main melting peak. This step tells the software where the peak is that you want to fit.
9. The curve Fitting Session window should be displayed. Pull that window to the right side of the screen so that you can see the thermogram.
10. The peak maximum is indicated with a vertical green line. If this line is not centered on your peak, estimate the peak maximum and type this temperature into the first parameter dialog box, which is labeled Tm. Also uncheck the "Vary?" checkbox to the right of the Tm parameter window, so that the curve fitting procedure will not vary this parameter.
11. Click on the 1 iteration button, at the bottom of the curve fitting window, several times. The red curve fit peak should begin to approach the shape of the thermogram.
12. When the fit line starts to closely fit the experimental data, click on the "Vary?" checkbox to the right of the Tm parameter box. Then finish the curve fitting procedure by clicking several times on the 100 iteration button. Note the curve fit values. If either of the enthalpy values are negative, delete the negative sign and refit the peak. Note the values of the parameters and the uncertainties. Click on Done to close the Fitting Session window. Click on the go-away box on the Script window.

13. Print the thermogram. The fit values will be included on the printout.

Excel: The curve fitting procedures using the software are very robust, however, the automatic procedure hides some of the ideas in this lab. Therefore, you will also determine ΔH_{cal} and ΔH_{vH} using Excel for one (and only one) of your runs. Follow the following steps:

1. In Origin pull down the Window menu and click on the window labeled by your original file name. A spreadsheet should appear with the baseline corrected data listed in columns.

- Export the data to an ASCII file using the File/Export option. Use the default settings for the file export. Use a new floppy disk to transfer your data to a PC that has Excel.
- Start a new spreadsheet in Excel. Use the File/Open menu to read in your data from the floppy disk. The default import options will work fine. The imported file should be loaded into two columns in your new spreadsheet.
- Use your Origin plot to choose the starting point for integrating the main peak. Choose a temperature that corresponds to a flat region of the baseline just before the main melting peak. Also note the temperature when the main melting peak returns essentially to the baseline.
- Integrate the peak in your Excel spreadsheet between the two temperatures that you chose in the last step. Hint: In the first row corresponding to the starting temperature place a "0". For example this cell might be C264. In the next cell immediately below (i.e. C265) type in " $=C264+B265*(A265-A264)$ ". Of course you will need to adjust the row numbers for your spreadsheet. Fill this formula down until you are at the final temperature that you chose for the end of the integral. The value of this cell is ΔH_{cal} .
- Calculate ΔH_{vH} from Eq. 7. (Remember to convert to Kelvin degrees. Also the spreadsheet values are in cal, not kcal) Both enthalpy values should be similar to the automatic curve fitting values. Calculate C.U. from your Excel values.
- Make a plot of C_p and ΔH vs. T.

Sample Cell Cleaning:

Using the needle guide and the long-needle syringe remove the sample. Wash the sample cell several times with ~0.75 mL portions of reagent grade water. Remove the water from both the sample and reference cell. Clean out the syringes with water rinses. Remember to turn off the bath on the rotary evaporator.

Report:

Report your values for values for ΔH_{cal} , ΔS , ΔH_{vH} , T_m , $\Delta T_{1/2}$, and C.U. including the uncertainties from Origin. Report the same values obtained from your Excel spreadsheet for one of your runs. Include your Origin and Excel plots. Explain why the formula in step 5 above produces the integral. From another group, obtain the results for the PC series member that you did not run.

Explain what occurs during the gel to liquid-crystalline phase transition of lipid bilayers.^{3,9,10} How does this differ from melting of the pure phospholipids (not in an aqueous suspension)? What is occurring structurally in the bilayer? Compare T_m 's for the phase transitions from the different phospholipid samples. Explain the trends you observe. Compare your values of ΔH_{cal} , ΔH_{vH} , ΔS , $\Delta T_{1/2}$ and C.U. within the series. What can you determine about the heat and entropy required for each transition? Why does it increase (or decrease) within the series? Discuss reasons for the trends you see in each series of experiments and explain it in terms of bilayer and phospholipid structure. You may want to refer to the website¹⁰ to learn more about the gel to liquid-crystalline phase transition.

Literature Cited:

1. S. M. Ohline, M. L. Campbell, M. T. Turnbull, and S. J. Kohler, "Differential Scanning Calorimetric Study of Bilayer Membrane Phase Transitions: A Biophysical Chemistry Experiment," *J.Chem Ed.*, **2001**, 78(9), 1251-6.
2. T. M. Koyama, C. R. Stevens, E. J. Borda, K. J. Grobe, and D. A. Cleary, "Characterizing the Gel to Liquid Crystal Transition in Lipid-Bilayer Model Systems," *Chem. Educator* **1999**, 4, 12-15.
3. D. Chapman, *Quarterly Reviews of Biophysics*, **1975**, 8, pp. 185-235.
4. Szoka, R.; Papahadjopoulos, D. *Annu. Rev. Biophys. Bioeng*, **1980**, 9, 467.
5. It should be pointed out that this interpretation of the gel to liquid-crystalline phase transition in proteins is controversial. The exact details of the phase transition are currently a hot topic of debate.²
6. Mason, J. *Methods Enzymol.* **1998**, 295, 468-494.
7. "DSC Data Analysis in Origin: Tutorial Guide, Vers. 7", Microcal, Inc., Northhampton, MA, **2002**, 89-91.
8. J. M. Sturtevant, "Biochemical Applications of Differential Scanning Calorimetry," in Ann. Rev. Phys. Chem., H. L. Strauss, G. T. Babcock, C. B. Moore, Eds., Annual Reviews, Inc, Palo Alto, CA, 1987, 38, 466-476.
9. Cevc, G.; Marsh, D. *Phospholipid Bilayers: Physical Principles and Models*; Wiley: New York, 1987; pp 2-48.
10. For images, see *Membranes*; <http://www.wellesley.edu/Chemistry/chem227/lipids/membranes.htm>.