Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 3963–3967, July 1982
Biochemistry

A scanning calorimetric study of small molecule–lipid bilayer mixtures

(ideal solutions/phase transitions/scanning calorimetry/solid solutions)

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Contributed by Julian M. Sturtevant, March 29, 1982

ABSTRACT **The influence of small molecules on the main phase transition of dimyristoyl phosphatidylcholine was observed by high-sensitivity differential scanning calorimetry. The variation with temperature of the excess heat capacity is markedly affected by solid solution formation, which appears to be of frequent occurrence in solutions in lipid bilayers. The experimental results are in some cases in reasonable agreement with ideal solution theory.**

The transfer of small molecules from aqueous medium into lipid bilayers is a process that is undoubtedly important in biological membranes and has accordingly received much attention in recent years (1–19). As discussed by Diamond and Katz (5) and Milgram and Solomon (20), this transfer involves rate processes in addition to the equilibrium distribution of solute between aqueous and lipid phases. In this paper I consider only the equilibrium distribution involving bilayers formed with pure dimyristoyl phosphatidylcholine (Myr_2-PtdCho). It must be kept in mind that, as recently shown by Conrad and Singer (21), much of the transfer data that have been obtained with lipid bilayers may have little or no relevance to protein-containing biological membranes.

Differential scanning calorimetric (DSC) evidence has been presented (22) which indicates that the gel-to-liquid-crystal transition, the so-called main transition, of phosphatidylcholines in multilamellar suspensions (liposomes) is a first-order transition. With perfectly ordered bilayers formed from pure lipids this transition would thus be isothermal. However, experimental limitations such as imperfect ordering of the lipid molecules in the bilayer, finite scan rates and calorimetric lags, and the presence of traces of impurities lead to observed transitions that extend over a finite range of temperature. The broadening can usually be approximately expressed in terms of the van't Hoff equation

$$\frac{d \ln\left(\frac{1-\alpha}{\alpha}\right)}{dT} = \frac{\Delta H_{vH}}{RT^2}, \qquad [1]$$

in which α is the extent of the phase transition at temperature T, ΔH_{vH} is the van't Hoff enthalpy, and R is the gas constant. Although this enthalpy in the case of lipid bilayers is much larger than the true, or calorimetric, enthalpy, ΔH_{cal}, because of intermolecular cooperation, it is nevertheless finite. In actual fact, experimental DSC curves always have more gradual changes in excess heat capacity

$$C_{ex} = \Delta H_{cal} \frac{d\alpha}{dT} \qquad [2]$$

near the start and the finish of a lipid phase transition than expected on the basis of Eq. 1. This can be attributed to the existence of a distribution of values for ΔH_{vH}, including some relatively small ones.

It has frequently been observed that the phase transitions of phospholipids are markedly affected by the presence of various solutes. It is the purpose of this paper to explore the information concerning solutes in lipid bilayers that can be obtained from a detailed analysis of the results of DSC experiments. I shall first outline the effects to be expected with solutes that form ideal solutions in both the gel and liquid crystal phases of lipids, and I shall then consider several examples, some of which appear to fit this development and some that obviously deviate from it.

Ideal Solution Theory. Mastrangelo and Dornte (23) developed an expression for the broadening of the melting transition of a pure substance caused by a solute forming ideal solutions in both solid and liquid phases. If it is assumed that this solute broadening and the van't Hoff broadening mentioned above are additive, one obtains the equation

$$\frac{T_0}{T} = 1 + RT_0\left(\frac{1}{\Delta H_{vH}} \ln\left(\frac{1-\alpha}{\alpha}\right) - \frac{\ln X_1}{\Delta H_{cal}} \frac{1}{\frac{K}{1-K} + \alpha}\right). \qquad [3]$$

Here T_0 is the temperature at which the phase transition of the "pure" lipid (lipid containing no added solute) is half completed, X_1 is the total mole fraction of lipid in the lipid phases, and K is the equilibrium constant for the distribution of solute between gel and liquid crystal phases. It is, of course, assumed that equilibrium in the distribution of solute between the lipid phases is maintained during the transition. It is also assumed that the constant K and the partitioning of solute between aqueous and lipid phases do not change appreciably within the narrow temperature range of the transition. Perhaps the weakest assumption involved here is that the bilayer behaves as an isotropic solvent, whereas it is known to be composed of oriented molecules and to vary from highly polar in the region of the head groups to highly apolar in the hydrocarbon interior (24). It may be argued that van't Hoff broadening takes place relative to the temperature of half transition in the presence of the solute rather than to T_0. This distinction produces no significant change in calculated values of T.

ΔH_{vH} for a "pure" lipid may be estimated by means of the equation

$$\Delta H_{vH} = 4RT_0^2 \frac{C_{ex,\,max}}{\Delta H_{cal}} \qquad [4]$$

or by less exact expressions derived from this equation, such

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Abbreviations: Myr_2-PtdCho, dimyristoyl phosphatidylcholine; DSC, differential scanning calorimetry.

as

$$\Delta H_{vH} \approx 6.9T_0^2/\Delta T_{1/2}. \quad [5]$$

In these expressions $C_{ex,\,max}$ is the maximal value of the excess heat capacity and $\Delta T_{1/2}$ is the width in degrees of the C_{ex} *vs* T curve, the DSC output, at half maximal C_{ex}. $C_{ex,\,max}$ and ΔH_{cal} in Eq. 4 can be expressed in any convenient units such that their ratio has the dimension of reciprocal temperature. It is to be expected that ΔH_{vH} in the presence of a solute may differ in either direction from ΔH_{vH} for the pure lipid.

Ideal solution theory has been applied by several authors to the partitioning of small molecules between aqueous and lipid phases but without inclusion of the formation of solid solutions. This omission can lead to seriously erroneous conclusions. In two publications (10, 13), the effect of solid solution formation on the transition temperature itself was considered. The experiments to be reported here and in future publications make it appear that solid solution formation is more prevalent with lipid bilayers than with ordinary solvents, perhaps because of the quasi-two-dimensional nature of the bilayer.

Fig. 1 shows the very pronounced effect that the existence of solid solutions has on the shape of a transition. The curves in Fig. 1 were calculated taking T_0 = 298.15 K, ΔH_{cal} = 5,000 cal mol^{-1}, $\Delta H_{vH} = 2 \times 10^6$ cal mol^{-1}, and the mole fraction of solute in the lipid, $X_2 = 1 - X_1$, equal to 0.02 (1 cal = 4.184 J). Curve A is for the case of K = 1, or alternatively X_2 = 0, and curves B, C, D, and E are for K = 0.8, 0.5, 0.3, and 0, respectively. Curves for which the Ks are the reciprocals of the values used in constructing Fig. 1 would be approximately the mirror images of the curves shown. It may be noted that an ideal solute for which K = 1 can have no influence on the transition, and that solute broadening similar to that shown in Fig. 1 will appear even with an indefinitely large ΔH_{vH}—that is, with a perfectly cooperative transition. In other words, an added solute may increase the width of the temperature range over which gel and liquid crystal phases coexist, but this can take place without any loss in transition cooperativity. Mountcastle *et al.*

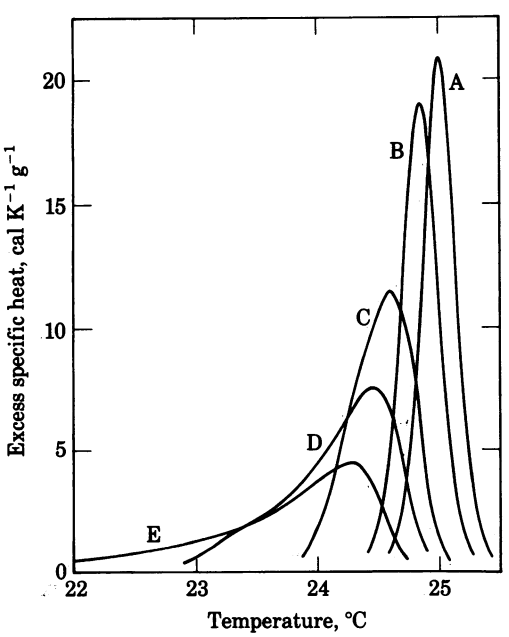

FIG. 1. Variation of excess specific heat with temperature as calculated according to Eq. 3 for T_0 = 298.15 K, ΔH_{cal} = 5,000 cal mol^{-1}, and $\Delta H_{vH} = 2 \times 10^6$ cal mol^{-1} for various values of X_2 and K (see text for definition of terms). Curve A, K = 1 or X_2 = 0; curves B–E, X_2 = 0.02 and K = 0.8, 0.5, 0.3, and 0, respectively.

(14) observed transition curves with shapes similar to those shown in Fig. 1 for liposomes of dipalmitoyl phosphatidylcholine containing small concentrations of inhalation anesthetics.

MATERIALS AND METHODS

Myr_2-PtdCho was purchased from Calbiochem (lot 810031) and was used as received except for dehydration at 40°C in a vacuum oven for several hours. Ethanol (95%), 1-butanol (99%), ethyl acetate, and potassium chloride were analytical grade reagents. Ultrapure urea was purchased from Bethesda Research Laboratories. Dodecane and hexadecane were gifts from Humphrey Chemical Company (North Haven, CT) and were specified to be at least 99% normal isomer. Doubly deionized water was used.

Preparation of Lipid Suspensions. Weighed amounts of solid lipid were suspended in 0.15 M KCl by vigorous mixing on a Vortex mixer for 30–60 sec at 45–50°C. Appropriate amounts of solute, either as liquid (dodecane and hexadecane) or as solutions in 0.15 M KCl, were added to known volumes of lipid suspension in small vials together with a few glass beads. Most of the samples were placed in a 40°C bath immediately after preparation and were gently agitated for at least 24 hr. The samples containing dodecane and hexadecane, and six of the samples containing ethyl acetate, were heated to 50°C for several minutes with two 30-sec periods of mixing before being placed in the 40°C bath. It was observed that these ethyl acetate samples on being scanned immediately after mixing gave traces significantly sharper (ΔT = 0.20 ± 0.01°C) than those given by samples that had been in the 40°C bath for 24 hr or longer, including the initially mixed samples ($\Delta T_{1/2}$ = 0.33 ± 0.02°C). This broadening, which took place with no significant change in t_m, the temperature of maximal excess specific heat, or in ΔH_{cal}, and thus appeared to be due to a decrease in ΔH_{vH}, was not systematically investigated in the present work. The use of unbuffered samples gave the possibility of detecting very small amounts of lysolecithin formation during incubation, because one proton is liberated for each lipid molecule hydrolyzed. No significant change in pH occurred after as long as 3 days at 40°C. Final lipid concentrations of 0.5 to 1.0 mg ml^{-1} were employed.

Calorimetry. All suspensions were scanned at 0.25 K min^{-1} in a DASM-1M scanning microcalorimeter (25), except for one sample containing 1-butanol, which was scanned at both 0.25 and 0.05 K min^{-1} to check on the maintenance of equilibrium during the scan. The lowering of t_m and the decrease in $\Delta T_{1/2}$ observed at the lower scan rate were about the same as observed with the pure lipid. Fig. 2 is a tracing of a typical DSC output curve and illustrates the good signal-to-noise ratio achieved throughout this work. The large pulse is a calibrating signal produced by feeding approximately 250 μW of extra power to the reference cell. This scan was obtained with a sample of Myr_2-PtdCho containing ethyl acetate. It is immediately evident on comparing this scan, showing a 1 K lowering of t_m, with the curves in Fig. 1 that solid solutions play an important role in this system.

RESULTS AND DISCUSSION

A number of solutes added to liposomes of Myr_2-PtdCho have been investigated. In this report I will restrict attention to potassium chloride, dodecane, hexadecane, ethanol, ethyl acetate, 1-butanol, and urea.

Potassium Chloride. It may be safely assumed that this solute is totally excluded from the hydrocarbon portion of the bilayer. It produces a small increase in both t_m and $\Delta T_{1/2}$, the values for Myr_2-PtdCho suspended in water and in 1.50 M KCl being

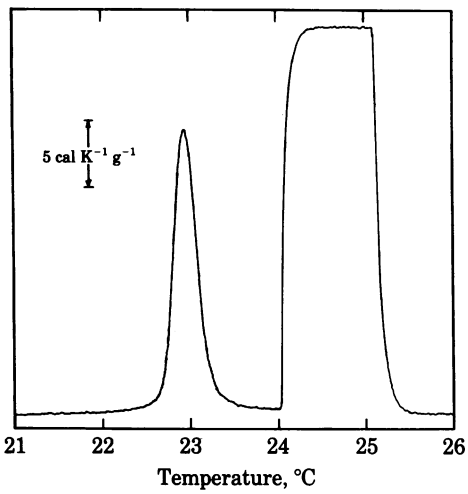

FIG. 2. Tracing of a typical DSC output curve, illustrating the general level of signal-to-noise ratio achieved in this work. The sample scanned was multilamellar Myr_2-PtdCho at a concentration of 0.525 mg ml^{-1} containing ethyl acetate at an overall concentration of 3.75 mg ml^{-1}. The scan rate was 0.25 K min^{-1}. The rectangular calibration signal was produced by supplying 15.96 mcal of excess energy to the reference cell over a period of 260 sec.

24.00 and 24.48°C, and 0.19 and 0.21°C, respectively. There was no significant effect on ΔH_{cal}.

Dodecane and Hexadecane. It is interesting that these two solutes, which are presumably totally excluded from the head group region, also raise t_m. Fig. 3 shows a smoothed tracing of a curve obtained with a small concentration of hexadecane present and with points calculated by using Eq. 3 with $T_0 = 297.05$ K, $\Delta H_{cal} = 5{,}195$ cal mol^{-1} (the observed value), $\Delta H_{vH} = 2 \times 10^6$ cal mol^{-1}, $X_2 = 0.02$, and $K = 4.5$. It seems quite clear that hexadecane is several times more soluble in the gel phase than in the liquid crystal phase, a surprising result. The value for X_2 together with the approximately known amount of hexadecane added indicate that hexadecane was present in the aqueous phase at a concentration of 13 ppm, presumably the solubility limit for the hydrocarbon.

A similar result was obtained with dodecane, though with a somewhat less satisfactory fit of the calculated points to the observed curve. In this case a value for K of about 2.6 is indicated.

Variation of t_m with Solute Concentration. Of the four remaining solutes listed above, three—ethanol, ethyl acetate, and 1-butanol—lower t_m, and one—urea—has no detectable effect on t_m. The variation of t_m with overall solute concentration is shown in Fig. 4. For the first three solutes, the least-squares slopes are in the ratios 1:5.7:11.3, whereas the partition coefficients between Myr_2-PtdCho liposomes and 0.15 M KCl at 25°C given by Katz and Diamond (4) are in the ratios 1:5.7:7.2. The fact that urea, with a lipid/KCl solution partition coefficient half that of ethanol (4), has no effect on t_m even at a calculated concentration in the lipid of 22 mg g^{-1} (mole fraction 0.2) strongly suggests that this solute is completely excluded from the hydrocarbon portion of the lipid bilayer.

The parameters for the least-squares lines in Fig. 4 and the respective standard deviations, along with the mean enthalpy of transition observed with each solute, are given in Table 1. The close adherence of the t_ms to linear dependence on concentration suggests that details of the solute–lipid interaction remain constant over the entire concentration range studied. The fact that the transition enthalpies are independent of solute concentration shows that any deviations from ideality are the same in both the gel and liquid crystal states.

Ethyl Acetate. The partition coefficient of ethyl acetate between Myr_2-PtdCho liposomes and 0.15 M KCl was measured by Katz and Diamond (4) over a range of temperatures below and above the main phase transition temperature. van't Hoff plots of these data give for the standard enthalpy of transfer from aqueous to lipid phase the values 27.2 kcal mol^{-1} below and 1.8 kcal mol^{-1} above the phase transition temperature. This enormous difference in enthalpy should be apparent in transition enthalpies as observed in DSC because any solute present is transferred from gel to liquid crystal phase during the transition,

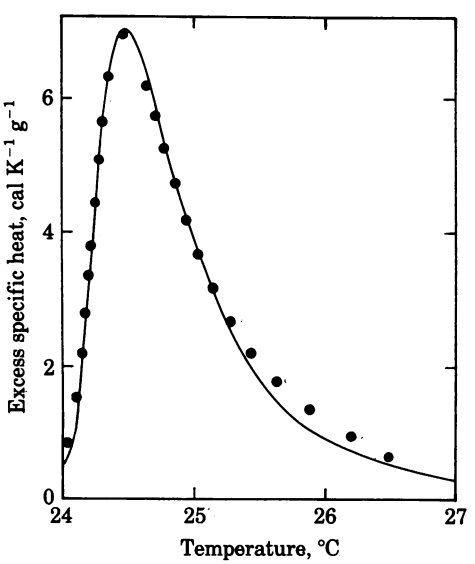

FIG. 3. Main transition of multilamellar Myr_2-PtdCho at a concentration of 1.37 mg ml^{-1} containing *n*-hexadecane at an overall concentration of approximately 0.022 mg ml^{-1}. The solid curve is the smoothed DSC output and the filled circles are points calculated according to Eq. 3 with $T_0 = 297.05$ K, $\Delta H_{cal} = 5{,}195$ cal mol^{-1}, $\Delta H_{vH} = 2 \times 10^6$ cal mol^{-1}, $X_2 = 0.02$, and $K = 4.5$.

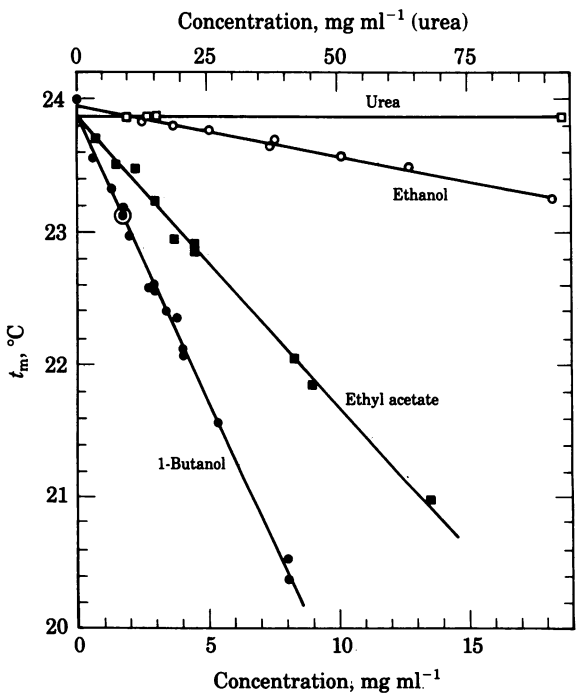

FIG. 4. Effect of added solutes on t_m, the temperature of maximal excess heat capacity, for the main transition of multilamellar Myr_2-PtdCho. t_m is plotted against the overall concentration of solute. The constants of the least-squares lines are given in Table 1.

Table 1. Concentration dependence of t_m and enthalpies of transition

Solute	a,* °C	b,* °C mg^{-1} ml	SD	ΔH, cal mol^{-1}
1-Butanol	23.86 ± 0.02	−(0.428 ± 0.006)	±0.06	5,420 ± 60
Ethyl acetate	23.86 ± 0.02	−(0.218 ± 0.006)	±0.05	5,170 ± 100
Ethanol	23.95 ± 0.01	−(0.038 ± 0.002)	±0.02	5,220 ± 130
Urea	23.87 ± 0.01	0	—	4,740 ± 80

ΔH is given as mean ± SEM, per mole of lipid.
* Constants in the equation $t_m = a + bc$, in which c is the overall concentration of solute in mg ml^{-1}. Values are mean ± SEM.

with an expected enthalpy change in this case of −25.4 kcal per mol of solute. No such changes were detected. For example, an experiment with ethyl acetate present at an overall concentration of 16.00 mg ml^{-1} showed t_m = 20.44°C. The data of Katz and Diamond (4) give 1.17 for the partition coefficient at this temperature, so that the solute concentration in the lipid is calculated to be approximately 18.7 mg g^{-1}, corresponding to a mole fraction of 0.128. Thus the observed transition enthalpy per mole of lipid should have been 4.82 ± 0.14 (the mean value for Myr_2-PtdCho observed in this work) − (0.128/0.872) × 25.4, or 1.99 kcal mol^{-1}. The actually observed value was 5.47 kcal mol^{-1}. The mean transition enthalpy observed in the 10 experiments with ethyl acetate shown in Fig. 3 was 5.20 ± 0.11 kcal mol^{-1}.

The three solutes studied by Katz and Diamond at temperatures below the transition temperature were found to have enthalpies of transfer from gel to liquid crystal state of −20.7 kcal mol^{-1} (butyramide), −25.4 kcal mol^{-1} (ethyl acetate), and approximately −21 kcal mol^{-1} (acetone). If we assume 1-butanol to be characterized by a value of −20 kcal mol^{-1}, at the highest concentration of this solute, 8.04 mg ml^{-1} overall, the transition enthalpy should have been about 1.1 kcal mol^{-1} instead of the actually observed value of 5.62 kcal mol^{-1}.

It is difficult to account for this striking discrepancy. My colleague, James Prestegard (personal communication), has suggested that, although it is not easy to visualize, insertion of solute molecules into the gel phase may be a cooperative process, with an average cooperative unit in the case of ethyl acetate of approximately 15 solute molecules.

The solid curve in Fig. 5 is a smoothed scan obtained in the presence of ethyl acetate at 3.75 mg ml^{-1} and Myr_2-PtdCho at 0.525 mg ml^{-1}. The data of Katz and Diamond (4) lead to a calculated mole fraction of ethyl acetate in the lipid of approximately 0.048. If this value is used in Eq. 4 with $K = 0$ and $\Delta H_{vH} = 2 \times 10^6$ cal mol^{-1}, a very broad transition curve with maximal C_{ex} of only 2.8 cal^{-1} g^{-1} at 22.2°C is calculated. Again it is seen that solid solutions are of importance in this system, as is also indicated by the partition coefficients given by Katz and Diamond (4). The observed DSC curve cannot be fitted by Eq. 4 with $X_2 = 0.048$ regardless of how large a value is assigned to ΔH_{vH}. The dashed curve is constructed by using $\Delta H_{vH} = 4 \times 10^6$ cal mol^{-1} and $K = 0.50$. This latter value was selected to place t_m approximately correctly. Reasonably good fits can be obtained with various values of the parameters; the filled circles in Fig. 5, for example, are points calculated by using $\Delta H_{vH} = 4 \times 10^6$, $X_2 = 0.12$, and $K = 0.80$. The fact that essentially equally good fits can be obtained with other values for the adjustable parameters means that the present approach cannot in all cases be employed to obtain accurate values for X_2 and K.

In view of the polarity gradient in the bilayer (24), it seems likely that polar solutes such as ethyl acetate are not uniformly

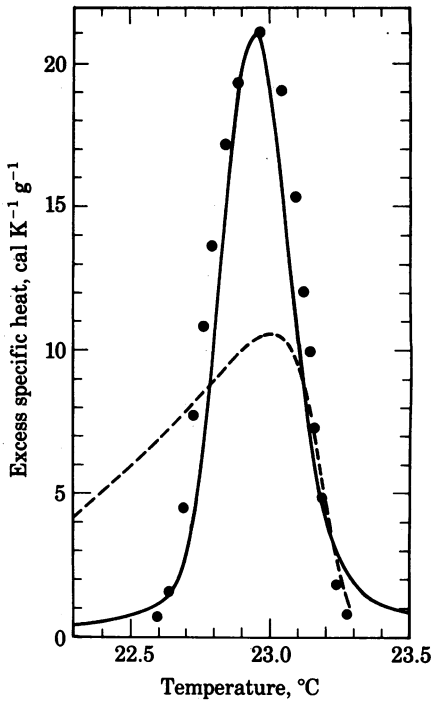

FIG. 5. DSC scan of multilamellar Myr_2-PtdCho (0.525 mg ml^{-1}) in the presence of ethyl acetate (3.75 mg ml^{-1} overall). The solid curve is the smoothed observed output. The dashed curve is constructed according to Eq. 3, using T_0 = 297.12 K, ΔH_{cal} = 5,380 cal mol^{-1} (the observed value), $\Delta H_{vH} = 4 \times 10^6$ cal mol^{-1}, $X_2 = 0.048$ [calculated from the partition coefficient given by Katz and Diamond (4)], and $K = 0.50$. The filled circles are calculated with $X_2 = 0.12$ and $K = 0.80$.

distributed in the bilayer but are concentrated in the head group region. Because the transition temperature of the bilayer is strongly dependent on the van der Waals interactions between the hydrocarbon tails, it might be expected that at a given average concentration a solute concentrated in the polar region would have less effect on the transition temperature than one that is distributed throughout the nonpolar hydrocarbon region. Actually, if we take the partition coefficient given by Katz and Diamond (4) to be correct, the case of ethyl acetate seems to work in the opposite direction; the solute has a much greater effect than its average concentration would lead one to expect.

Ethanol and 1-Butanol. These solutes gave DSC curves quite similar to those observed with ethyl acetate. The sharp transitions observed even with large lowerings of t_m clearly indicate again the importance of solid solutions. As in the case of ethyl acetate, it is impossible to fit the experimental data by means of Eq. 4 with values for X_2 calculated on the basis of the partition coefficients given by Katz and Diamond (4), considerably larger values for X_2 being required.

Although it happened that none of the solutes used in this study had any significant effect on the enthalpy of transition, this is certainly not always the case. Preliminary experiments with Myr_2-PtdCho containing a Ca^{2+}-blocking drug, nitrendipine, kindly supplied by Robert Colvin of the University of Connecticut Health Center, showed that there is a very large negative enthalpy of transfer of the drug between the gel and liquid crystal phases. A mole fraction of the drug in the lipid of less than 0.1 decreased the transition enthalpy to 700 cal mol^{-1}!

CONCLUSIONS

High-sensitivity DSC has revealed the ubiquitous occurrence of solid solutions when small molecules are dissolved in lipo-

somes of Myr_2-PtdCho and presumably of other phospholipids. The shape of a DSC curve gives at least qualitative information concerning the concentration of the solute in the lipid and its partitioning between the gel and liquid crystal phases of the lipid. The transition enthalpy gives a value for the enthalpy of transfer between the two phases; when this is significantly different from zero, it can be concluded that there is a more marked deviation from ideality in one phase than in the other.

I am indebted for helpful discussions to Drs. Barbur Chowdhry, Adam Dalziel, and Gert Lipka. This research was aided by grants from the National Institutes of Health (GM 04725) and the National Science Foundation (PCM 7824107).

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