MULTICOMPONENT PHASE TRANSITIONS OF DIACYLPHOSPHATIDYLETHANOLAMINE DISPERSIONS

BABUR Z. CHOWDHRY, GERT LIPKA, ADAM W. DALZIEL, AND JULIAN M. STURTEVANT
Department of Chemistry, Yale University, New Haven, Connecticut 06511

ABSTRACT The phase transition properties of aqueous suspensions of a series of nonhydrated (not heated above room temperature) and hydrated 1,2 diacylphosphatidylethanolamines (PE's) have been examined by high sensitivity differential scanning calorimetry at scan rates of 0.02–1.0 K min^{-1}. At all scan rates nonhydrated PE's show a single asymmetric transition curve of excess heat capacity as a function of temperature. Multilamellar dispersions of hydrated PE's, however, exhibit transitions with fine structure, which can be fitted as the sum of three two-state component transitions, at scan rates of 0.02–0.1 K min^{-1}, but give only a single asymmetric transition at 1.0 K min^{-1}. At all scan rates the transition(s) of hydrated samples occur at lower temperatures than those of nonhydrated samples. One of the component transitions of hydrated PE's may be analogous to the pretransition that occurs in 1,2 diacylphosphatidylcholines.

INTRODUCTION

Diacylphosphatidylethanolamines (PE's) are widely distributed in the membranes of both procaryotic (1) and eucaroytic cells (2). The curves of excess apparent specific heat vs. temperature for the gel-to-liquid crystalline transition of multilamellar liposomes of PE's reported to date (3–7), and upon which specific theoretical analyses for the physical behavior of PE's have been partly based (8, 9), display a transition that is asymmetrically broadened toward lower temperatures. In contrast to the complex phase behavior of diacylphosphatidylcholine (PC) bilayers (10), it has appeared that there are no transitions at temperatures below that of the gel-to-liquid crystalline transition of fully hydrated PE's (3–7). These differences have been attributed to the difference in the head group between PE's (RN^+H_3) and PC's ($R\text{-}N^+[CH_3]_3$), and resulting differences in hydrogen bonding capacity, electrostatic effects, hydration properties, and acyl-chain packing (11, 12). Examination of dilauroyl, dimyristoyl, dipalmitoyl, and distearoyl PE's by high sensitivity differential scanning calorimetry (HSDSC) reveals, however, that the phase transitions of PE's are more complex than previously realized.

The calorimetrically detectable phase transition properties of multilamellar phospholipid vesicles are dependent upon purity and concentration of lipid, method of preparation, acyl-chain length, and the nature of the head group (13). The apparent values of the thermodynamic parameters for the transitions may be affected by instrumental characteristics, such as heat transfer rates, and it is therefore important to employ low scan rates (e.g., 0.02–0.1 K min^{-1}) in calorimetric studies of lipid transitions (14). Although reports exist of the phase transition properties of PC's at low scan rates (14), no such data have been reported for hydrated PE's of high purity.

MATERIALS AND METHODS

Lipid Preparations

All phospholipids were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Their purity was checked by thin layer chromatography (TLC) in different solvent systems (15) and by gas liquid chromatography (GLC; 16) and found to be at least 99.5%. Before use all lipids were kept in a vacuum oven at 50°C until no further weight loss occurred. Nonhydrated suspensions were prepared by addition of doubly deionized water to weighed lipids to give concentrations in the range 1.0–5.0 mg ml^{-1}, folowed by vortexing at room temperature for 3 min. The lipid dispersion was immersed for several minutes in an acetone/solid CO_2 bath and then allowed to thaw. The vortexing and freeze-thaw procedure was repeated three times. The term nonhydrated samples is taken to mean suspended samples of lipids that have not been heated above room temperature (23°C). Multilamellar liposomes were prepared by the addition of 5 ml of doubly deionized water to 2–10 mg of phospholipid, heating to 10°C above the phase transition temperature (T_{NH}) of nonhydrated samples and vortexing for 30 s. The suspension was reheated and vortexed three times. Alternatively multilamellar liposomes of fully hydrated PE's were also prepared by the method given for nonhydrated PE's followed by heating and cooling samples, in the calorimeter, to 10°C above and below T_{NH} three times, at a heating rate of 2 K min^{-1}. The calorimetric properties of multilamellar vesicles produced by these two methods did not differ significantly when scanned at 0.1 K min^{-1}.

Calorimetry

DSC experiments were performed in a DASM-1M microcalorimeter (17) using scan rates of 0.02–1.0 K min^{-1}. Instrumental baselines obtained by scanning doubly deionized water in both sample and reference cells of the calorimeter were horizontal in the range 0–80°C. The initial and final baselines for all curves of apparent excess specific heat vs. temperature (C vs. T) were also horizontal with no significant changes in apparent excess specific heat. The noise level was in the range 0.01–0.02 cal K^{-1} gm^{-1}.

After the first calorimetric scan of multilamellar dispersions, the samples were cooled in the calorimeter and then rescanned to check on the reproducibility and reversibility of the transitions. All samples were run under an excess nitrogen pressure of 1.5 atm to minimize bubble formation.

Data Analysis

Multicomponent transition curves were resolved into the sums of independent component curves, which were required to follow the van't Hoff equation for a simple two-state process (18). For this purpose a computer program was employed (19, 20), which varied in succession the three parameters that characterize each van't Hoff curve, until the standard deviation of the calculated sum curve from the observed data was minimal. The parameters used were the calorimetric enthalpy (ΔH_{cal}, proportional to the area of the transition curve), the van't Hoff enthalpy (ΔH_{vH}, which controls the variation with temperature of the equilibrium constant appropriate to the curve), and the temperature of half completion of the transition (T_H). The sum of the values of ΔH_{cal} for the resolved components agreed to within ±1–2% with ΔH^T_{cal}, the total observed enthalpy evaluated by planimeter integration (21) of the curves of C vs. T.

RESULTS AND DISCUSSION

Nonhydrated samples of PE's show a single asymmetric transition with a broad low temperature side followed by a steeper high temperature side (Fig. 1). The transition temperatures (T_{NH}) are higher than the transition temperatures (T_H) of hydrated samples (Fig. 1 and Table I). The difference between T_{NH} and T_H decreases with increasing chain length (inset, Fig. 1); extrapolation indicates that these two temperatures coincide at C_{20} chain length. The

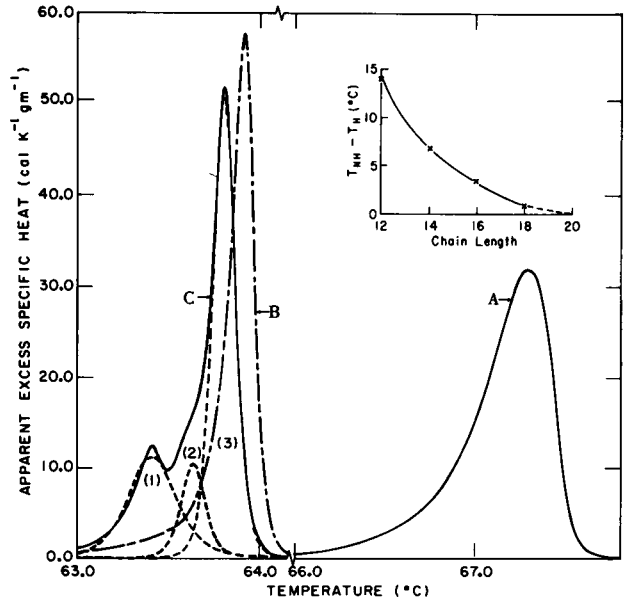

FIGURE 1 Curves of apparent excess specific heat vs. temperature for dipalmitoylphosphatidylethanolamine. (*A*) Nonhydrated sample scanned at 0.1 K min^{-1}; (*B*) hydrated sample scanned at 1 K min^{-1}; (*C*) hydrated sample scanned at 0.1 K min^{-1} (—, experimental curve, ---, component transition curves). The lipid concentration was 1 mg ml^{-1} in all cases. The inset shows the difference in the temperature of maximal specific heat between nonhydrated (T_{NH}) and hydrated (T_H) samples.

transition shown by nonhydrated samples has been attributed to simultaneous hydration of head groups and acyl-chain melting (22). D. A. Wilkinson and J. F. Nagle (manuscript submitted for publication) have ascribed this transition to the melting of a more ordered, less hydrated, subgel phase, which is stable over its entire temperature range (0–57°C for DMPE), with formation of the liquid crystalline phase. A similar interpretation has been given by Mantsch et al. (22) and by Chang and Epand (23). Seddon et al. (24) have also reported, for DLPE, transitions at 30.6°C and 43°C and an additional one at ~37°, which has not been seen by any other workers. The half height width ($\Delta T_{1/2}$), calorimetric enthalpy (ΔH_{cal}), maximal apparent excess specific heat (C_{max}), and van't Hoff enthalpy (ΔH_{vH}) are similar for all the nonhydrated PE's studied here. However, the values we have found for T_{NH} and C_{max} are higher and values for $\Delta T_{1/2}$ are much smaller than those previously reported (22–24) (Table I). These differences probably reflect the higher purity of the PE's used in this study and the low scan rates employed.

In contrast to nonhydrated samples, hydrated dispersions of PE's show multicomponent transitions (Fig. 1 *C*). The component transitions are not as well resolved at scan rates of 0.25–0.5 K min^{-1} as at 0.1 K min^{-1}. At 1.0 K min^{-1} a single asymmetric transition curve is obtained similar to those reported in the literature for PE's (Fig. 1 *B*). The transition curves for hydrated PE's, as observed at a scan rate of 0.1 K min^{-1}, can be analyzed as the sum of three two-state or van't Hoff curves. Although this analysis is convenient for the purpose of describing our observations, it does not necessarily give a uniquely valid interpretation. However, the clear occurrence of inflexions in the transition curves gives added confidence that curve fitting to three transitions is a valid procedure.

That the component peaks are not due to contamination of the PE's is shown by a variety of observations, including analytical TLC (7, 15) and GLC (16) before and after DSC scans, DSC examination of hydrated samples before and after preparative TLC and/or recrystallization, and after purification of PE samples from a different supplier (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). DSC study of PE samples to which the corresponding lyso PE's were added shows that the multicomponent phase behavior is not due to this type of contamination. Rescanning dispersions of hydrated PE's several times over periods of 1–12 h after the first scan showed no changes in the scan profiles. Centrifugation (10^5 *g* for 1 h) of hydrated samples followed by calorimetric examination of supernatant and of precipitate resuspended by gentle shaking, as well as negative staining electron microscopy, indicated that the samples consisted entirely of multilamellar vesicles. No transition due to hydrated phospholipid was observed using nonhydrated samples. Similarly, when hydrated material was scanned, no transition due to nonhydrated material could be seen, indicating

TABLE I
PHASE TRANSITION PROPERTIES OF NONHYDRATED AND HYDRATED PHOSPHOLIPIDS

	Nonhydrated				
	$T_{(NH)}$	$\Delta t_{1/2}$	ΔH_{cal}	ΔH_{vH}	C_{max}
	°C	°C	kcal mol^{-1}	kcal mol^{-1}	cal k^{-1} gm^{-1}
Phospholipid	(±0.02)	(±0.02)	(±0.2–0.4)	(±45)	(±0.5–2.0)
Dilauroyl PE	44.65	0.46	13.8	1,500	30.8
Reference 22	44.0	1.3*	13.3	—	—
Reference 23	43.2	1.7*	10.0	—	10.0*
Reference 24	43.0	2.3*	13.6	—	10.0*
Dimyristoyl PE	56.80	0.45	13.3	1,700	31.0
Reference 22	53.20	2.2*	14.8	—	—
‡	56.30	0.6	16(±0.6)	—	—
Dipalmitoyl PE	67.30	0.44	12.7	1,800	32.0
Reference 22	64.90	1.8*	18.5	—	—
Distearoyl PE	75.20	0.44	12.9	1,900	30.5

	Hydrated					
	T_H	$\Delta t_{1/2}$	ΔH_{cal}	ΔH^T_{cal}	C_{max}	ΔH_{vH}
	°C	°C	kcal mol^{-1}	kcal mol^{-1} (±0.1–0.4)	cal k^{-1} gm^{-1}	kcal mol^{-1}
Phospholipid						
Dilauroyl PE (1)	29.91	0.33	1.1	—	3.8	1,900
Dilauroyl PE (2)	30.25	0.24	1.3	4.80	8.5	2,700
Dilauroyl PE (3)	30.44	0.16	2.4	—	23.5	4,000
Reference 22	30.5	1.0*	—	—	—	—
Reference 23	30.1	0.33*	2.5	2.5	8.5*	—
Reference 24	30.6	0.9	3.7	3.7	5.0*	—
Dimyristoyl PE (1)	49.40	0.40	2.85	—	5.62	1,800
Dimyristoyl PE (2)	49.70	0.10	0.63	8.43	8.7	7,000
Dimyristoyl PE (3)	49.94	0.13	4.95	—	41.3	5,600
Reference 22	49.0	0.9*	—	—	—	—
‡	49.6	0.3	5.7(±0.1)	5.7	24.0	—
Dipalmitoyl PE (1)	63.40	0.31	3.12	—	11.1	2,600
Dipalmitoyl PE (2)	63.66	0.22	1.76	10.08	10.4	3,600
Dipalmitoyl PE (3)	63.80	0.13	5.20	—	50.8	6,100
Reference 22	63.2	0.9*	—	—	—	—
Dipalmitoyl PE*	63.90	0.15	8.50	—	57.0	5,300
Distearoyl PE (1)	74.12	0.34	3.50	—	15.4	2,500
Distearoyl PE (2)	74.36	0.27	2.30	11.4	10.6	3,100
Distearoyl PE (3)	74.40	0.18	5.55	—	44.0	4,700
DPPC (PT)	34.80	0.90	1.35	—	1.9	740
DPPC (GLCT)	41.50	0.11	7.60	—	74.0	6,300

Our data are for samples scanned at 0.1 K min^{-1} in doubly deionized water except for dipalmitoyl PE* scanned at 1.0 K min^{-1} (see Fig. 1 *B*). For comparison, the phase transition properties of the pretransition (PT) and gel-to-liquid crystalline transition (GLCT) of 1,2 dipalmitoylphosphatidylcholine (DPPC) are given. The DPPC dispersions were prepared in exactly the same way as the hydrated PE samples.

*Estimated value from data given in the indicated reference.

‡D. A. Wilkinson and J. F. Nagle (manuscript submitted for publication).

that the phospholipid had been properly hydrated. All these results indicate clearly that the multicomponent phase behavior of PE's is an intrinsic property of the lipids.

We propose that in each case the component transition occurring at the highest temperature (transition 3, Fig. 1) is due primarily to the cooperative melting of the alkyl chains, and may thus be identified with the so-called main (gel-to-liquid crystalline) transition of PC's (5, 18). The value of C_{max} for transition 3 is in all cases considerably higher than that previously reported (3–7) presumably because of the higher purity of the lipids used in our work, and the value of $\Delta T_{1/2}$ is comparable to those reported for the main transitions of high purity PC's (5, 18).

We also propose that one of the two lower transitions (transition 1 or 2, Fig. 1) is analogous to the so-called pretransition of PC's. Other observations are consistent with this hypothesis. For example, it has been shown (25) on the basis of ^{13}C and ^{2}H NMR studies that during the gel-to-liquid crystalline transition of PE's, changes in rotational motion about the molecular axis, similar to those associated with the pretransition in PC's, take place. It has

also been inferred (26) from ESR measurements that an increase in axial rotational motion takes place at temperatures coinciding with or immediately below the pretransition in PC's and the gel-to-liquid crystalline transition of PE's. In addition, it has recently been demonstrated that in PC's a pretransition occurs both in the presence and absence of lanthanum ions (27), despite the fact that the acyl-chain tilt angle relative to the normal to the bilayer plane is 0° in the presence of lanthanum and 30° in its absence (28). It has been previously suggested that PE's do not undergo a pretransition because their acyl chains are orientated perpendicular to the bilayer plane (11, 12). This assumption may not be correct in view of the results obtained with the lecithin-lanthanum system (27). Interestingly, the temperature of the pretransition of PC's increases in the presence of long chain alkanes (C_{14}—C_{16}) until at a mole ratio of PC/alkane of 1:2 (a ratio at which the acyl chains are perpendicular to the bilayer plane 28) the pretransition merges with the gel-to-liquid crystalline transition (B. Z. Chowdhry, unpublished observations). It is not known however, which one of the lower temperature component transitions of the PE's may be a pretransition, and we have no explanation for the existence of the other component. A possibility is that component transitions 1 and/or 2 represent changes in the state of hydration of PE's. It will be very difficult to examine the nature of the component transitions by physicochemical methods other than HSDSC because of the very small temperature differences between them.

We thank D. Keene for assistance with electron microscopy.

This research was supported in part by research grants from the National Institutes of Health (GM04725) and the National Science Foundation (PCM-7824107).

Received for publication 24 August 1983 and in final form 15 December 1983.

REFERENCES

1. Overath, P., and L. Thilo. 1978. Structural and functional aspects of biological membranes revealed by lipid phase transitions. *Int. Rev. Biochem.* 19:1–44.
2. Rothman, J. E., and J. Lenard. 1977. Membrane symmetry. *Science (Wash. DC).* 195:743–753.
3. Mabrey, S., and J. M. Sturtevant. 1976. Investigation of phase transitions of lipids and lipid mixtures by high sensitivity differential scanning calorimetry. *Proc. Natl. Acad. Sci. USA.* 73:3862–3866.
4. Blume, A. 1980. Thermotropic behavior of phosphatidylethanolamine-cholesterol and phosphatidylethanolamine-phosphatidylcholine-cholesterol mixtures. *Biochemistry.* 19:4908–4913.
5. Wilkinson, D. A., and J. F. Nagle. 1981. Dilatometry and calorimetry of saturated phosphatidylethanolamine dispersions. *Biochemistry.* 20:187–192.
6. Chapman, D., J. Urbina, and K. M. Keough. 1974. Biomembrane phase transitions (studies of lipid-water systems using differential scanning calorimetry). *J Biol. Chem.* 249:2512–2521.
7. Vaughan, D. J., and K. M. Keough. 1974. Changes in phase transitions of phosphatidylethanolamine and phosphatidylcholine-water dispersions induced by small modifications in the head group and backbone regions. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 47:158–161.
8. Berde, C. B., H. C. Andersen, and B. S. Hudson. 1980. A theory of the effects of head group structure and chain unsaturation on the chain melting transition of phospholipid dispersions. *Biochemistry.* 19:4279–4293.
9. Nagle, J. F. 1980. Theory of the main lipid bilayer phase transition. *Annu. Rev. Phys. Chem.* 31:157–195.
10. Stumpel, J., H. Eibl, and A. Niksch. 1983. X-ray analysis and calorimetry of phosphatidylcholine model membranes (the influence of length and position of acyl chains upon structure and phase behavior). *Biochim. Biophys. Acta.* 727:246–254.
11. Rand, R. P., D. Chapman, and K. Larsson. 1975. Tilted hydrocarbon chains of dipalmitoyl lecithin become perpendicular to the bilayer before melting. *Biophys. J.* 15:1117–1124.
12. Pearce, P. A., and H. L. Scott. 1982. Statistical mechanics of the ripple phase in lipid bilayers. *J. Chem. Phys.* 77:951–958.
13. McElhaney, R. N. 1982. The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes. *Chem. Phys. Lipids.* 30:229–259.
14. Albon, N., and J. M. Sturtevant. 1978. Nature of the gel to liquid crystal transition of synthetic phosphatidylcholines. *Proc. Natl. Acad. Sci. USA.* 75:2258–2260.
15. Christie, W. W. 1982. Lipid Analysis. Second ed. Pergamon Press, New York. 107–153.
16. Sheppard, A. J., and J. L. Iverson. 1975. Esterification of fatty acids for gas liquid chromatographic analysis. *J Chromatogr. Sci.* 13:448–470.
17. Privalov, P. L. 1980. Scanning microcalorimeters for studying macromolecules. *Pure Appl. Chem.* 52:449–497.
18. Mabrey, S., and J. M. Sturtevant. 1978. High sensitivity differential scanning calorimetry in the study of biomembranes and related model systems. *Methods Membr. Biol.* 9:237–274.
19. Epand, R. M., L. T. Boni, and S. W. Hui. 1982. Interaction of glucagon with sphingomyelins. *Biochim. Biophys. Acta.* 692:330–338.
20. Spink, C. H., K. Müller, and J. M. Sturtevant. 1982. Precision scanning calorimetry of bile salt phosphatidylcholine micelles. *Biochemistry.* 24:6598–6605.
21. Velicelebi, G., and J. M. Sturtevant. 1979. Thermodynamics of the denaturation of lysozyme in alcohol-water mixtures. *Biochemistry.* 18:1180–1186.
22. Mantsch, H. H., S. C. Hsi, K. W. Butler, and D. G. Cameron. 1983. Studies on the thermotropic behaviour of aqueous phosphatidylethanolamines. *Biochim. Biophys. Acta.* 728:325–330.
23. Chang, H., and R. M. Epand. 1983. The existence of a highly ordered phase in fully hydrated dilauroylphosphatidylethanolamine. *Biochim. Biophys. Acta.* 728:319–324.
24. Seddon, J. M., K. Harlos, and D. Marsh. 1983. Metastability and polymorphism in the gel and fluid bilayer phases of dilauroylphosphatidylethanolamine. *J. Biol. Chem.* 258:3850–3854.
25. Blume, A., D. M. Rice, R. J. Wittenbort, and R. G. Griffin. 1982. Molecular dynamics and conformation in the gel and liquid crystalline phases of phosphatidylethanolamine bilayers. *Biochemistry.* 21:6620–6230.
26. Marsh, D. 1980. Molecular motion in phospholipid bilayers in the gel phase: long axis rotation. *Biochemistry.* 19:1632–1637.
27. Chowdhry, B. Z., G. Lipka, A. Dalziel, and J. M. Sturtevant. 1984. Effect of lanthanum ions on the phase transitions of lecithin bilayers. *Biophys. J.* 45:633–635.
28. McIntosh, T. J. 1980. Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphadtidylcholine bilayers; a molecular packing model. *Biophys. J.* 29:237–246.