Apparent Molar Heat Capacities of Phospholipids in Aqueous Dispersion. Effects of Chain Length and Head Group Structure†

Alfred Blume

ABSTRACT: Apparent molar heat capacities $^{\phi}C_p$ of a variety of different phospholipids in dilute aqueous dispersion were determined by using high-sensitivity differential scanning calorimetry. The apparent molar heat capacities vary with head group structure and chain length in a systematic way. The experimental $^{\phi}C_p$ values are higher than those estimated from group parameter values for a lipid bilayer where only the head groups and the glycerol backbone are hydrated. Thus positive contributions to the apparent molar heat capacities arising from "hydrophobic hydration" are probable. The same conclusion is reached when a lipid in a micellar state, i.e., lysolecithin, is compared with bilayer-forming lipids. These results suggest that more water than previously estimated may be present in hydrophobic parts of the bilayer. This could explain the anomalously high water and H^+/OH^- permeability of phospholipid bilayers.

For the investigation of the thermodynamic properties of phospholipid bilayers, differential scanning calorimetry has become a standard method for its ease of operation and for the advantage of monitoring the intrinsic properties of the bilayer. Until recently, all calorimetric investigations have focused on the determination of transition temperatures and enthalpies of the gel to liquid-crystalline phase transition of lipid bilayers [for reviews, see Lee (1977) and Nagle (1980) and references cited therein] and not much was known about the thermodynamic properties of the single-phase regions due to the limited sensitivity and base-line stability of the available instruments. Only the structural and dynamical properties of these phases were determined by techniques such as X-ray diffraction (Hitchcock et al., 1974; Janaik et al., 1979), NMR[1] measurements (Seelig, 1977), dilatometry (Melchior & Morowitz, 1972; Nagle, 1973; Nagle & Wilkinson, 1978), capacitance measurements (Ashcroft et al., 1981), and a variety of probe methods. The first authors to report on specific heats of lipids in the single-phase regions were Wilkinson & Nagle (1982), who used a Microcal MC-1 calorimeter with lipid concentrations of 16–36 mg·mL^{-1}. The specific heat of phospholipids was found to be considerably higher than that of alkanes and to display strong temperature dependence for gel-phase lipids. Wilkinson and Nagle suggested an unhindering of chain vibrations as a cause for this phenomenon.

In view of the wealth of information on structure and dynamics and the scarcity of data on the thermodynamic properties of these phases, a detailed analysis of several phospholipid classes seemed desirable. An important aspect in this study was to test whether "hydrophobic hydration" in bilayers plays a role and how bilayers are related to micellar systems (Tanford, 1973). The availability of a highly sensitive differential scanning calorimeter with excellent base-line reproducibility (Privalov et al., 1975) made it possible to determine apparent molar heat capacities of dilute suspensions (1 mg·mL^{-1}) of phospholipids with sufficient accuracy.

In this paper, apparent molar heat capacities $^{\phi}C_p$ of three different phospholipid classes, namely, PCs, PEs, and PAs, will be presented. The experimental $^{\phi}C_p$ values are higher than expected from calculations with $^{\phi}C_p$ group parameter values under the assumption that only the head groups and the glycerol backbone are hydrated. $^{\phi}C_p$ depends on the nature of the phospholipid head group and displays a linear increase with chain length. This increase in $^{\phi}C_p$ is larger than expected for methylene groups in a pure hydrophobic environment. On the basis of these results, contributions of hydrophobic hydration to the apparent molar heat capacities will be discussed.

Materials and Methods

The phospholipids DMPC, DPPC, DSPC, DLPE, DMPE, DPPE, DSPE, DAPE, DMPA, DPPA, DHPC, DHPE, and LPC were purchased from FLUKA, Neu-Ulm. DAPC was a product from Paesel, Frankfurt, and DTPA and DHPA were gifts from Dr. H. Eibl, Göttingen. All lipids were chromatographically pure as tested by TLC on silica gel plates (Merck, Darmstadt) and used without further purification.

Multilamellar liposome suspensions were prepared by suspending the desired amount of lipid in bidistilled water above the respective phase-transition temperature T_m and vortexing the suspension for 1–2 min. In some cases, i.e., with long-chain PEs, additional short sonication (30 s) with low energy in a Branson bath-type sonicator was employed. The lipid samples were equilibrated at temperatures above the respective T_m for 30 min before loading them into the calorimetric cell. Lipid concentrations were generally 1 mg·mL^{-1} (ca. 1.5 mM). In some cases, i.e., with DMPC and DPPC, higher concentrations (2–4 mg·mL^{-1}) were also tested. The exact phospholipid concentration was determined after the calorimetric runs from the original sample by using a modified procedure for phosphate analysis (Hague & Bright, 1941). Concentration determinations from separate aliquots of the sample varied in general within ±0.8% except for PEs where due to the coarseness of the dispersions, variations were somewhat greater

† From the Institut für Physikalische Chemie II, D-7800 Freiburg, West Germany. *Received February 16, 1983.* This work was supported by the Deutsche Forschungsgemeinschaft.

[1] Abbreviations: $^{\phi}C_p$, apparent molar heat capacity; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; LPC, 1-palmitoyllysolecithin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine; DLPE, dilauroylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; DAPE, diarachidoylphosphatidylethanolamine; DMPA, dimyristoylphosphatidic acid; DPPA, dipalmitoylphosphatidic acid; DTPA, ditetradecylphosphatidic acid; DHPC, dihexadecylphosphatidylcholine; DHPE, dihexadecylphosphatidylethanolamine; DHPA, dihexadecylphosphatidic acid; T_m, transition temperature; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

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(±2%). Differential scanning calorimetry was performed in a Privalov DASM-1 calorimeter (Privalov et al., 1975). The effective cell volume was 0.98 mL. A heating rate of 1 K·min^{-1} was used for all experiments. At this scan rate, the transition peaks are slightly broadened due to the slow instrument response. However, this has no effect on the transition enthalpies. For the determination of the apparent molar heat capacities, this scan rate gave optimum results. The calorimeter was connected to a home-built 64K solid-state memory (Messner, 1978). The stored calorimetric scans were subsequently read into a Hewlett-Packard 9845A computer for further data processing.

$^{\phi}C_p$ values were calculated from the shift of the base line recorded when the sample cell contained the lipid dispersion, relative to a water/water base line with the procedure described by Privalov & Khechinashvili (1974):

$$^{\phi}C_p = \left(c_{p,W}\frac{V_L}{V_W} - \frac{\Delta}{m_L} \right) M_L$$

with M_L = molecular weight of lipid, $c_{p,W}$ = specific heat of water, V_L and V_W = specific volume of lipid and water, respectively, m_L = mass of lipid in sample cell, and Δ = displacement of the base line relative to a water/water base line in calories per kelvin.

Thus for the determination of $^{\phi}C_p$, the specific volume V_L of the phospholipid must be known. V_L values for PCs and PEs have recently been determined by dilatometry (Nagle & Wilkinson, 1978; Wilkinson & Nagle, 1981). The specific volumes are in the range between 0.9 and 0.96 mL·g^{-1} for gel-phase lipids. They increase roughly by 0.1%·K^{-1}. V_W is also temperature dependent, but considerably less, i.e., 0.035%·K^{-1} (Weast, 1981). The change in the specific heat of water in the temperature range between 0 and 100 °C is very small and can be neglected. For the calculation of $^{\phi}C_p$, we used a fixed value for $c_{p,W}(V_L/V_W)$ over the whole temperature range. The error in $^{\phi}C_p$ due to this approximation is ca. ±2% for gel-phase lipids. For lipids in the liquid-crystalline phase, the $^{\phi}C_p$ values shown in the calorimetric scans in the figures are consistently too low by ca. 3–4% as the increase in specific volume at the phase transition was neglected in the calculation. The temperature dependence of V_L could have been easily included in the calculation of $^{\phi}C_p$, but at the present stage, we did not think it to be worthwhile, as the overall precision in determining $^{\phi}C_p$ still needs to be improved (see below).

The main error in the determination of $^{\phi}C_p$ stems from the variations in the base line after consecutive scans and, what is even more important, after the cells are refilled. With a standardized filling procedure, we were able to reduce the variations in the absolute value of the base line after consecutive refills to ±0.025 mcal·K^{-1}. The general shape of the base line in the temperature range between 5 and 95 °C is even better reproducible. The overall accuracy in determining $^{\phi}C_p$ values, including the approximations for V_L, can thus be estimated to ±30 cal·K^{-1}·mol^{-1} or ±5–10% when lipid concentrations of 1 mg·mL^{-1} are used. In principle, the accuracy should improve with higher lipid concentrations. However, experiments with concentrations of 2 or 4 mg·mL^{-1} did not give better results, mainly for two reasons: (1) the error in the concentration determination became larger due to an increase in the inhomogeneity of the dispersion; (2) we observed larger variations in the absolute values of the calorimetric scans upon reheating the samples. Usually the curves shifted to lower $^{\phi}C_p$ values with increasing number of scans and then reached a plateau. The source of this phenomenon could not be determined. At concentrations of 1 mg·mL^{-1}, the variations in the scans were within the normal range. Each sample was generally scanned 2–3 times. The reproducibility of the scans was usually very good and the calculated $^{\phi}C_p$ values varied within the estimated error limits.

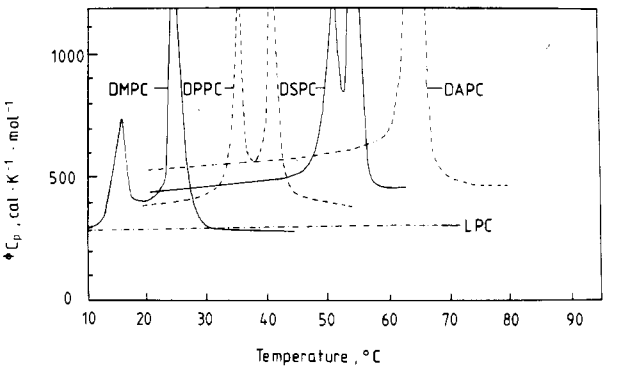

FIGURE 1: Apparent molar heat capacities $^{\phi}C_p$ of aqueous dispersions of phosphatidylcholines and of lysolecithin micelles as a function of temperature.

Results

Figure 1 shows calculated $^{\phi}C_p$ curves for multilamellar dispersions of four different PCs. The ordinate scale has been expanded so the peaks of the main transitional endotherms are off scale. The $^{\phi}C_p$ values for small unilamellar vesicles may be different as vesicles are known to give broader transitions with lower transition enthalpies (Gruenewald et al., 1979). However, vesicles tend to aggregate and fuse to larger multilamellar structures so that $^{\phi}C_p$ values for these systems cannot be determined.

With increasing chain length, the main transition is shifted to higher temperature, and the temperature difference between the pre and main transition decreases (Chapman, 1968). $^{\phi}C_p$ between these two transitions in the $P_{\beta'}$ phase is higher than in the $L_{\beta'}$ or L_{α} phase. This was first observed by Hinz & Sturtevant (1972) and is particularly evident in the scans of DMPC and DPPC. In DSPC, both transitions start to overlap, and for DAPC only a shoulder ca. 2 deg below the maximum of the main peak can be seen (not visible in Figure 1, as the maximum of the shoulder is at 1360 cal·K^{-1}·mol^{-1}). All PC samples were scanned after short equilibration at lower temperature, so the highly ordered subphase could not form (Chen et al., 1980). The $^{\phi}C_p$ values clearly increase with chain length as can be seen from the displacement of the curves. Also included in this figure is a $^{\phi}C_p$ curve of 1-palmitoyllysolecithin, a lipid in the micellar state at this particular concentration (Lewis & Gottlieb, 1971). The specific volume for LPC was assumed to be 1 mL·g^{-1}. This approximation seems reasonable as experimentally determined specific volumes of surfactant micelles are in the range from 0.9 to 1.1 mL·g^{-1} (Musbally et al., 1974; Desnoyers et al., 1980).

Figure 2 shows $^{\phi}C_p$ curves for five different PEs. The fatty acyl chains of PEs in the gel phase are oriented perpendicular to the bilayer surface (McIntosh, 1980), so only one endothermic transition from the L_{β} to the L_{α} phase is observed. The transitions of PEs are broader than for the corresponding PCs, and the width increases for the longer chain analogues. The $^{\phi}C_p$ behavior in the single-phase regions is similar to that observed before in PCs. Again, $^{\phi}C_p$ clearly shows a chain-length dependence.

The phase behavior of phosphatidic acids can be modified by changing the external pH or the ionic strength of the dispersion. Increasing the pH to 12 results in the dissociation of the second proton, and the head group becomes doubly

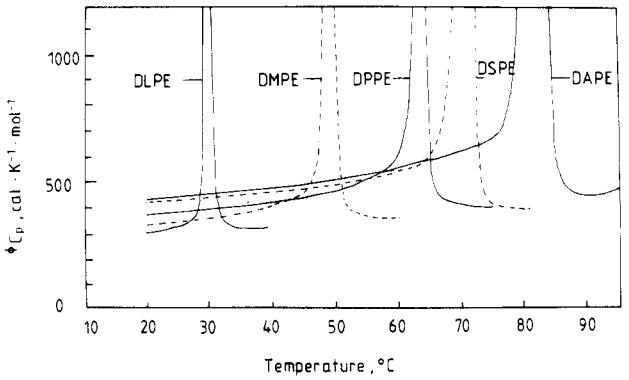

FIGURE 2: Apparent molar heat capacities $^{\phi}C_p$ of aqueous dispersions of phosphatidylethanolamines as a function of temperature.

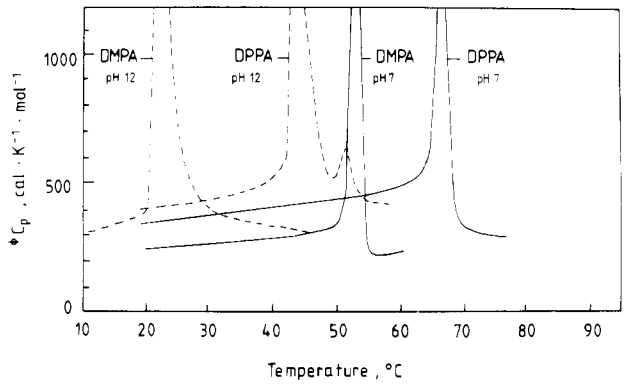

FIGURE 3: Apparent molar heat capacities $^{\phi}C_p$ of aqueous dispersions of phosphatidic acids in the singly (pH 6) and doubly (pH 12) charged form as a function of temperature.

Table I: Apparent Molar Heat Capacities $^{\phi}C_p$ (cal·K^{-1}·mol^{-1}) and Specific Heats c_p (cal·K^{-1}·g^{-1}) of Lipids with Palmitoyl Chains[a]

	T = 20 °C		ΔT = −20 °C		ΔT = 10 °C	
	$^{\phi}C_p$	c_p	$^{\phi}C_p$	c_p	$^{\phi}C_p$	c_p
DPPC	380	0.505	380	0.505	400	0.531
DPPE	375	0.542	430	0.621	390	0.564
DPPA (pH 6)	330	0.494	420	0.628	290	0.435
DPPA (pH 12)	390	0.563	410	0.591	420	0.607

[a] Data are average values of three runs. The total error in the specific heat is approximately ±0.035 cal·K^{-1}·g^{-1} or ±5–10% (see text).

charged (Träuble &Eibl, 1974). The increased electrostatic repulsion between the head groups leads to a change in the tilt angle of the chains and to a decrease of the transition temperature (Jähnig et al., 1979). Figure 3 shows $^{\phi}C_p$ curves for DMPA and DPPA in the singly and doubly charged form. At constant pH, $^{\phi}C_p$ increases again with chain length. Doubly charged PAs have higher apparent molar heat capacities than singly charged PAs. As PAs at pH 12 are chemically not stable over longer periods of time, each sample could only be scanned once or twice. The origin of the small endothermic peak on the high-temperature side of the transition of DPPA at pH 12 is unknown but could possibly arise from hydrolysis during the scan.

The apparent molar heat capacities $^{\phi}C_p$ as well as the specific heats c_p depend on the nature of the head group. Values for dipalmitoyl phospholipids at 20 °C and on a reduced temperature scale with ΔT = −20 and +10 °C are shown in Table I.

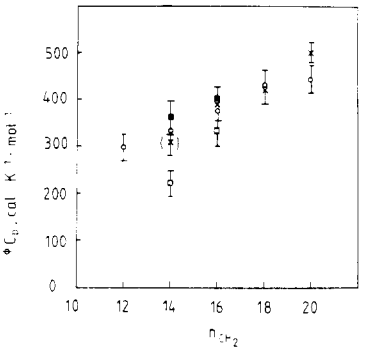

FIGURE 4: Apparent molar heat capacities $^{\phi}C_p$ at 20 °C for aqueous dispersions of phospholipids: (×) PC; (○) PE; (□) PA (pH 6); (■) PA (pH 12). $^{\phi}C_p$ for DMPC is for 10 °C, i.e., below the pretransition.

Table II: Average Increase in $^{\phi}C_p$ per Additional Methylene Group $\Delta^{\phi}C_p$ (cal·K^{-1}·mol^{-1}) for Phospholipids in the Gel and Liquid-Crystalline Phase in Comparison with Solid and Liquid n-Alkanes[a]

lipid	T = 20 °C	T = 80 °C
PC	13.0	16.5
PE	10.5	7.5
PA (pH 6)	(27.5)	(33)
PA (pH 12)	(10.0)	(24)
n-alkanes	4.5[b]	7.8[b]
hydrophobic hydration	21.5[c]	

[a] $\Delta^{\phi}C_p$ values for PAs are only tentative as only two compounds of this lipid class were investigated. [b] Data from Messerly et al. (1967). [c] Data from Roux et al. (1978).

In Figure 4, we have plotted the $^{\phi}C_p$ values for all three lipid classes as a function of chain length at T = 20 °C. An almost linear increase is obtained, and a least-squares fit leads to $\Delta^{\phi}C_p$ values per methylene group as shown in Table II. $\Delta^{\phi}C_p$ values for n-alkanes (Messerly et al., 1967) and for hydrophobic hydration (Roux et al., 1978) are also included for a comparison. The $\Delta^{\phi}C_p$ values for gel-phase lipids are higher than those of solid alkanes but lower than those for hydrophobic hydration. Similar results are obtained for lipids in the L_α phase at 80 °C, but in this case, the correlation of our data is not as good as some curves had to be extrapolated to higher temperature. However, with the exception of PEs, the $\Delta^{\phi}C_p$ values are again higher than those for liquid alkanes.

The apparent molar heat capacities of the ether analogues DHPC, DHPE, DHPA, and DTPA were almost identical with those of the ester lipids, though in some cases they seemed to be slightly lower. However, these differences are within the accuracy of our $^{\phi}C_p$ determination.

Table III summarizes the thermodynamic data for the pretransitions and main transitions of all phospholipids investigated in this study. The determination of the ΔH values for the pretransition and main transition of PCs is somewhat difficult as the $^{\phi}C_p$ curve does not return to the same value between the two transitions. We have arbitrarily chosen the following procedure: The area under the $^{\phi}C_p$ curve covering the temperature range of both transitions was divided at a temperature halfway between the two transitions. The two parts were then assigned to the pretransition and main transition, respectively. This procedure increases the ΔH values for DMPC and DPPC slightly, as the $^{\phi}C_p$ value does not return to the same value between the two transitions (Hinz & Sturtevant, 1972).

Inspection of Table III shows the following: (1) The transition enthalpy ΔH_2 for the main transition increases with chain length but not in a linear fashion. This is particularly evident in the case of PEs, where the difference in ΔH de-

Table III: Transition Temperatures for Pretransition (T_{m_1}) and Main Transition (T_{m_2}), Transition Enthalpies for Pretransition (ΔH_1) and Main Transition (ΔH_2), and Transition Entropies for Main Transition (ΔS_2) for Phospholipids in Dilute Aqueous Dispersion (c = 1 mg·mL^{-1})

lipid	T_{m_1} (°C)	T_{m_2} (°C)	ΔH_1 (kcal·mol^{-1})	ΔH_2 (kcal·mol^{-1})	ΔS_2 (cal·K^{-1}·mol^{-1})
DMPC	15.3	24.0	1.3	6.5	21.9
DPPC	35.5	41.5	1.6	8.7	27.7
DSPC	51.0	54.3	1.8	10.9	33.3
DAPC	62.1	64.1	1.7	12.3	37.6
DLPE		30.5		4.3	14.2
DMPE		49.9		6.6	20.4
DPPE		63.9		8.6	25.5
DSPE		70.4		10.5	30.6
DAPE		81.1		12.2	34.5
DMPA (pH 6)		52.2		5.7	17.5
DMPA (pH 12)		22.4		4.1	13.9
DPPA (pH 6)		65.0		7.9	23.4
DPPA (pH 12)		43.1		5.7	17.8
DHPC	33.0	43.5	1.4	8.5	26.7
DHPE		68.5		7.6	22.3
DTPA (pH 6)		63.5		4.6	13.7
DTPA (pH 12)		31.0		2.9	9.5
DHPA (pH 6)		73.3		7.1	20.5
DHPA (pH 12)		53.8		5.8	17.8

creases from 2.2 to 1.7 kcal·mol^{-1}. (2) The ΔH and ΔS values vary with the nature of the head group. The differences between PCs and PEs are not large, but PAs have definitely lower transition enthalpies and entropies in the singly as well as in the doubly charged form. (3) Ether analogues have higher transition temperatures but lower transition enthalpies. This effect is more pronounced for lipids with strong polar head group interactions, i.e., PEs and PAs.

Discussion

Absolute Values of Apparent Molar Heat Capacity $^{\phi}C_p$. There are several contributions to the heat capacity, and we want to divide them into two categories, the intrinsic heat capacity and the structural heat capacity. Contributions to the intrinsic heat capacity arise from molecular vibrations, from motions within the lattice, i.e., long-axis rotation and translational diffusion, and from the work of expansion, as the specific volume of the bilayer increases with temperature (Nagle & Wilkinson, 1978). The structural part of the heat capacity reflects the interactions of the lipid molecules with the surrounding water. For amphiphiles, these interactions can be hydrophilic or hydrophobic in nature. The interactions of hydrophobic molecules with water have been a field of intensive research since the pioneering work of Frank & Evans (1945). The enhancement of the hydrogen-bonded structure of liquid water around nonpolar groups leads to large negative contributions to the molar entropy and positive contributions to the heat capacity (Franks, 1975). In contrast, the partial molar heat capacities of simple monovalent salts as an example of hydrophilic hydration are all negative (Rüterjans et al., 1968). From $^{\phi}C_p$ measurements of various organic molecules with alkyl chains and different hydrophilic groups dissolved in water, it was found that the average increase in $^{\phi}C_p$ per additional methylene group is ~21.5 cal·K^{-1}·mol^{-1} (Rüterjans et al., 1968; Arnett et al., 1969; Nichols et al., 1976; Cabani et al., 1977; Roux et al., 1978). As a comparison, in solid and liquid *n*-alkanes the heat capacity increases only by 4.5 and 7.8 cal·K^{-1}·mol^{-1}, respectively (Messerly et al., 1967). Nichols et al. (1976) and Roux et al. (1978) devised a simple additivity scheme for approximating partial molar heat capacities of solutes at infinite dilution and reported $^{\phi}C_p$ group parameter values for several other besides CH_2 groups. The agreement between predicted and measured $^{\phi}C_p$ values was always quite satisfactory. Also, $^{\phi}C_p$ measurements of long-chain detergents like sodium dodecyl sulfate at infinite dilution and the $^{\phi}C_p$ changes upon micellization have been reported (Musbally et al., 1974; Desnoyers et al., 1980; Choudhury & Ahluwalia, 1982). The apparent molar heat capacities of these detergents can be predicted in the same way. Upon micelle formation, $^{\phi}C_p$ decreases rapidly because the hydrocarbon chains associate and hydrophobic hydration decreases (Desnoyers et al., 1980). However, this decrease in $^{\phi}C_p$ is smaller than expected if all apolar groups would be transferred to a pure hydrocarbon environment. The conclusion that some of the methylene groups of the chains must therefore still be in contact with water is supported by spectroscopic and other evidence. It was found that on the average four to seven methylene groups in micelles come in contact with water and that this number is practically independent of type of surfactant, i.e., the nature of the head group and the length of the alkyl chain (Kresheck, 1975; Menger et al., 1978; Menger, 1979). We have also measured $^{\phi}C_p$ of a phospholipid in the micellar state, i.e., LPC, which has a critical micelle concentration of $<1.2 \times 10^{-4}$ M and micellar aggregation number of ca. 180 (Lewis & Gottlieb, 1971). Thus, LPC micelles behave similar to other surfactant micelles. For a 2×10^{-3} M solution, we obtained a $^{\phi}C_p$ value of 300 ± 30 cal · K^{-1}·mol^{-1} at 20 °C (see Figure 1). As LPC micelles are similar to other detergents, we can assume that also for this system ca. five CH_2 groups are in contact with water, resulting in a positive contribution to $^{\phi}C_p$. Taking the experimental $^{\phi}C_p$ of LPC as a basis, we can now estimate $^{\phi}C_p$ for DPPC in the liquid-crystalline phase by using the following assumptions: (a) the hydration of the head group does not change, (b) the contribution of hydrophobic hydration of five CH_2 groups is removed in going to the bilayer, giving a change in $^{\phi}C_p$ of $5 \times -15 = -75$ cal·K^{-1}·mol^{-1}, and (c) the addition of the second chain can be approximated by adding the heat capacity of liquid pentadecane (Messerly et al., 1967) and one –COO– group and subtracting the group parameter value of one OH group [the group parameter values for –COO– and –OH were taken from Roux et al. (1978)]. This approximation yields a value of $300 - 75 + 112 - 12 - 2 = 323$ cal·K^{-1}·mol^{-1} for $^{\phi}C_p$ of DPPC in the liquid-crystalline phase. The experimental $^{\phi}C_p$ for DPPC at 50 °C is 420 cal·K^{-1}·mol^{-1} (including the correction for the volume increase at the transition). As the temperature dependence of $^{\phi}C_p$ in the liquid-crystalline phase over a 30 °C range is small, we find for the difference between experimental and estimated $^{\phi}C_p$ a value of ca. 90 cal·K^{-1}·mol^{-1} using this procedure.

A second way to calculate $^{\phi}C_p$ is to add all group parameter values for the hydrated head group and backbone up to the ester groups plus the molar heat capacity of two liquid hydrocarbon chains. This procedure is less reliable as the head group contributions have to be partly approximated by using $^{\phi}C_p$ values of ions (Friedman & Krishnan, 1975). With different group parameter values, one calculates $^{\phi}C_p$ values in the range between 250 and 320 cal·K^{-1}·mol^{-1}. So both methods consistently yield $^{\phi}C_p$ values lower than those experimentally determined. We interpret this difference as being due to a structural contribution to $^{\phi}C_p$ through hydrophobic hydration.

This difference would mean that five to six methylene groups are in contact with water.

At first glance, this seems rather unlikely. However, there is other independent evidence that water can reach the hydrocarbon part of the bilayer. Griffith et al. (1974) used ESR spin-labels to measure the polarity profile across membranes and concluded that water may penetrate one-third of the distance from the surface into the bilayer. However, the bulky polar spin-label group may induce some perturbation of the bilayer, so this result has to be viewed with caution. Neutron-diffraction studies of gel-state DPPC could show that water penetrates to at least both carbonyl groups of DPPC (Büldt et al., 1978; Zaccai et al., 1979). Because of the bent conformation of the acyl chain in the 2-position of the glycerol backbone (Hitchcock et al., 1974), the first three CH_2 groups of the 2-chain would then be "wetted". The dielectric thickness of bilayers can be determined by capacitance measurements. Recent measurements revealed an electrically distinct region other than the hydrophobic chain and polar head group region. This was assigned to the ester region. From the value of the dielectric constant, it was concluded that water may penetrate up to the second methylene group of both chains (Ashcroft et al., 1981). In a recent X-ray and capacitance study of PE/cholesterol bilayers, it was reported that water penetration is reduced upon cholesterol addition and that in pure PE bilayers, water can reach the deeper carbonyl group of the 1-chain (Simon et al., 1982). Finally, phospholipid membranes display high water and H^+/OH^- permeability. It was suggested that this could be caused by a transient network of hydrogen-bonded water molecules dissolved in the hydrophobic part of the bilayer (Lawaczeck, 1979; Nichols & Deamer, 1980; Rossignol et al., 1982; Elamrani & Blume, 1983). All these experimental findings suggest that there may be more water molecules dissolved in the hydrophobic part of the bilayer than commonly assumed. Our $^{\phi}C_p$ data point in the same direction and add further evidence to the importance of bilayer–water interactions.

For the approximation of $^{\phi}C_p$ for gel-state lipids, we would have to reduce the $^{\phi}C_p$ contributions from the chains, as solid alkanes have lower heat capacities, as well as the contributions from hydration of the head groups, as gel-phase lipids are less hydrated (Lis et al., 1982; Guldbrand et al., 1982). The difference between experimental and calculated values would then become even greater. It is unlikely that hydrophobic hydration in the gel phase is larger. So other contributions to $^{\phi}C_p$, i.e., from chain vibrations as suggested by Wilkinson & Nagle (1982), play an important role. This will be discussed below.

$^{\phi}C_p$ approximations for PEs would predict values that are 30 to 40 cal·K^{-1}·mol^{-1} lower because of the smaller head group of PE. However, compensating effects between hydrophobic and hydrophilic hydration may occur. We find no large differences between PCs and PEs except for the longer chain analogues. For singly charged PA, we would expect $^{\phi}C_p$ to be ca. 80 cal·K^{-1}·mol^{-1} lower than that for the corresponding PC. This agrees with our results for gel-state lipids (see Figure 4) and is also true for PA in the L_α phase. Unexpected are the generally higher $^{\phi}C_p$ values for doubly charged PA. One would calculate a decrease in $^{\phi}C_p$ as the doubly charged head group has a more negative $^{\phi}C_p$ contribution. However, the increase in charge leads to a structural rearrangement, i.e., an increased tilt of the chains (Jähnig et al., 1979). This structural change could be associated with increased hydrophobic hydration overcompensating the negative $^{\phi}C_p$ from the head group. The magnitude of this hydrophobic effect would have to be anomalously large, and we think this to be very unlikely. That this $^{\phi}C_p$ increase is no artifact is supported by an independent experiment, namely, from the temperature dependence of the heat of dissociation of the second proton of DMPA (A. Blume, unpublished results). A more plausible explanation is the assumption that counterion binding may play a role, i.e., that the displacement of water of hydration from the doubly charged phosphate group upon binding of sodium ions is responsible for the increase in $^{\phi}C_p$. For linear polyelectrolytes like double-stranded DNA, the fraction of ions condensed on the negatively charged phosphate groups is remarkably high, namely, 0.76 (Manning, 1969). In this case, the average spacing of the negative charges is 1.7 Å. When DNA is denatured, the spacing increases from 1.7 to 4.3 Å, and the fraction of condensed ions decreases to 0.39 (Record et al., 1976). In a two-dimensional PA bilayer, the average charge separation is ca. 7 Å. This is lower than in the linear system, but counterion condensation is likely to occur also, particularly when PA is in the doubly charged form. This assumption is supported by the results of Cevc et al. (1980), who found that the surface potential of phosphatidylglycerol bilayers is 50 mV lower than that calculated from the surface charge density. This difference could be attributed to counterion condensation. As mentioned above, counterion condensation should increase drastically for doubly charged PA. The released water of hydration could then account for the experimentally observed higher $^{\phi}C_p$ values of PAs at pH 12.

Chain-Length Dependence of $^{\phi}C_p$. A puzzling question is the chain-length dependence of $^{\phi}C_p$ as shown in Table II. For gel-phase lipids, we would expect an average increase of 4.5 cal·K^{-1}·mol^{-1} per additional CH_2 group when we take solid alkanes as a reference system. For the liquid-crystalline phase, this value should increase to 8 cal·K^{-1}·mol^{-1}. However, for gel-state lipids, $\Delta^{\phi}C_p$ per CH_2 group is always larger than for *n*-alkanes, with 10.5 and 13.0 cal·K^{-1}·mol^{-1} for PEs and PCs, respectively. This difference is even more pronounced when we compare these two lipids at the same reduced temperature. At $\Delta T = -20$ °C, we find $\Delta^{\phi}C_p$, values of 13.4 cal·K^{-1}·mol^{-1} (0.03 cal·K^{-1}·g^{-1}) for PEs and 25 cal·K^{-1}·mol^{-1} (0.046 cal·K^{-1}·g^{-1}) for PCs. In the L_α phase, only PEs display the expected behavior. For purely hydrophobic hydration, $\Delta^{\phi}C_p$ would be 21.5 cal·K^{-1}·mol^{-1} per CH_2 group (Nichols et al., 1976). It seems unlikely that hydrophobic hydration should increase with chain length. However, the differences in the $\Delta^{\phi}C_p$ values between PCs and PEs correlate with their different hydration behavior. Lis et al. (1982) have measured the hydration repulsion of bilayers of several different PCs and found a similar chain-length dependence. They also compared egg yolk PC with egg yolk PE and found lower hydration forces for the PE. This correlates with the known lower water uptake of PEs (Jendrasiak & Hasty, 1974). This dependence of hydration on chain length and head group structure seems to be reflected in the differences in the $\Delta^{\phi}C_p$ values, PEs giving lower $\Delta^{\phi}C_p$ values than the better hydrated PCs. The hydration behavior of PAs has not been investigated up to now. We measured only two compounds of this lipid class. This is not enough to justify a detailed comparison with other lipids. Therefore, the $\Delta^{\phi}C_p$ values in Table II are only tentative at the present stage.

Temperature Dependence of $^{\phi}C_p$. For a better impression of the temperature dependence of $^{\phi}C_p$, we have replotted the $^{\phi}C_p$ curves for PEs on a reduced temperature scale (see Figure 5). Also included is the $^{\phi}C_p$ curve for two octadecane chains in the solid and liquid state (Messerly et al., 1967). $^{\phi}C_p$ for PEs increases much more rapidly below T_m than the heat

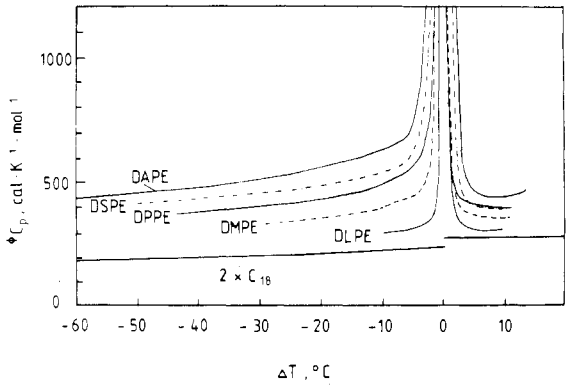

FIGURE 5: Apparent molar heat capacities $^{\Phi}C_p$ for phosphatidylethanolamines plotted on a reduced temperature scale: (ΔT) temperature difference $T_m - T$ (°C); (2 × C_{18}) temperature dependence of the heat capacity of 2 mol of *n*-octadecane (Messerly et al., 1967).

capacity of solid octadecane. Above the transition, $^{\Phi}C_p$ shows a precipitous decrease to much lower values than just below T_m, the difference getting larger with increasing chain length. These findings agree with the results obtained by Wilkinson & Nagle (1982). These authors used a Microcal MC-1 instrument with lipid concentrations of 16–36 mg·mL^{-1}. As mentioned above, we find differences in the absolute values for $^{\Phi}C_p$ (or specific heat) between the different phospholipid classes (see Table I). We also see differences in the temperature dependence of $^{\Phi}C_p$ between PCs and PEs. For instance, for DAPC $^{\Phi}C_p$ increases by 70 cal·K^{-1}·mol^{-1} (=13%), but for DAPE, the increase is larger, namely, 120 cal·K^{-1}·mol^{-1} (=26%) over a 30-deg temperature range (ΔT = −45 to −15 °C). There are also differences in the precipitous decrease of $^{\Phi}C_p$ above T_m between the different phospholipids. This shows that head group structure not only influences the absolute values of $^{\Phi}C_p$ but also its temperature dependence. Wilkinson & Nagle interpreted the increase in $^{\Phi}C_p$ for gel-phase lipids as being caused by an unhindering of chain vibrations as the lipids undergo thermal expansion and supported their hypothesis by approximate calculations. We would like to suggest that changes in head group hydration and intermolecular interactions and possibly increased contributions from hydrophobic hydration may also be important.

The hydration of the polar head group of phosphatidylcholines increases when the lipids expand on going through the main phase transition (Lis et al., 1982; Guldbrand et al., 1982). This should influence $^{\Phi}C_p$, though a priori no predictions can be made whether positive contributions from increased hydrophobic hydration overcompensate negative contributions arising from better hydration of the polar parts. In the case of PEs, an additional contribution could arise from changes in the extent of intermolecular hydrogen bonding between the head groups. In fact, PCs with the same chain length do not display such a large drop in $^{\Phi}C_p$ at the main transition as PEs (see Figures 1 and 2). PAs at pH 6 in the singly charged form behave similar to PEs in this respect, as PAs are also able to form intermolecular hydrogen bonds (Eibl & Wolley, 1979). Consequently, the decrease in $^{\Phi}C_p$ for doubly charged PAs is much less pronounced (see Figure 3). While all these findings give no direct measure for the magnitude of contributions arising from hydrophilic and hydrophobic hydration, they nevertheless indicate that changes in hydration have an influence on the absolute as well as the temperature dependence of the $^{\Phi}C_p$ values.

In the liquid-crystalline phase 10–15 deg above T_m, $^{\Phi}C_p$ does not change to such a large extent, and the temperature dependence is similar to that found for liquid alkanes. As suggested by Wilkinson & Nagle (1982), the generally lower heat capacities can be explained by the decrease in the coefficient of thermal expansion and the increased number of gauche conformations as most vibrations can occur freely.

One additional point we want to discuss is the higher $^{\Phi}C_p$ values of PCs in the intermediate $P_{\beta'}$ phase. For PCs with longer chains this is actually due to an overlap of both transitions. However, for DMPC and DPPC this is clearly not the case (see Figure 1). Recent ^{13}C and ^{2}H NMR experiments have shown that in the temperature range from just below the pretransition up to the main transition, the system is microscopically heterogeneous as two conformationally inequivalent lipid species can be detected, one growing with temperature at the expense of the other (Wittebort et al., 1981, 1982; Blume et al., 1982). One of the components shows some conformational characteristics of a lipid in the L_α phase, in particular, displaying much faster rotational diffusion. For DPPC bilayers ca. 3 deg above the midpoint of the pretransition peak, both components exist in equal proportions. This heterogeneity, especially the continually increasing proportion of the faster rotating component, may actually be the reason for the higher $^{\Phi}C_p$ values between pretransition and main transition. In a sense, one could speak of an unhindering of rotational degrees of freedom in addition to the proposed unhindering of chain vibrations taking place in this temperature interval.

Conclusions

High-sensitivity differential scanning calorimetry offers the possibility to determine apparent molar heat capacities of lipids in dilute aqueous dispersion. The absolute values of $^{\Phi}C_p$ as well as the relative changes with temperature, structure of the head group, and length of the hydrocarbon chain provide information about the thermodynamic properties of the different phases. Particularly, contributions from hydrophilic and hydrophobic hydration to $^{\Phi}C_p$ can be evaluated. The experimental apparent molar heat capacities are higher than the $^{\Phi}C_p$ values estimated by adding $^{\Phi}C_p$ group increments. These findings suggest that substantial contributions from hydrophobic hydration are responsible for the high $^{\Phi}C_p$ values. $^{\Phi}C_p$ was found to depend on the nature of the polar head group as well as on the length of the hydrocarbon chains. This phenomenon is not fully understood at the present time, though the dependence on head group structure seems to correlate with the hydration characteristics of the polar groups and/or the particular packing mode of the hydrocarbon chains. The same can be said for the temperature dependence of $^{\Phi}C_p$ in the gel phase and for the change of $^{\Phi}C_p$ at the phase transition. The increase of $^{\Phi}C_p$ in the gel phase is larger than that for solid alkanes. It likely arises from an unhindering of chain vibrations as suggested Wilkinson & Nagle (1982). However, temperature-induced changes in the hydration behavior could also contribut to the observed effects.

At the present stage, we are still far away from a quantitative understanding of the observed $^{\Phi}C_p$ values, and much experimental as well as theoretical work needs to be done to separate intrinsic $^{\Phi}C_p$ contributions from those arising from hydrophilic and hydrophobic hydration. The complexity of amphiphilic systems and possible compensating effects of the different $^{\Phi}C_p$ contributions complicates the analysis of the data. However, the observed high $^{\Phi}C_p$ values of phospholipids suggest that hydrophobic hydration is important and that more water than previously estimated may be present in the hydrophobic interior of the membrane. This would explain the anomalously high water and H^+/OH^- permeability of bilayer membranes.

In view of the wealth of experimental data on surfactant water interactions in micellar systems and the conflicting

conclusions drawn from the experimental data about micelle structure and water penetration into micelles (Kresheck, 1975; Menger, 1979; Wennerstrom & Lindman, 1979; Fromherz, 1981; Dill & Flory, 1981), the reported data for the apparent molar heat capacities of phospholipids can also be interpreted in different ways. Our interpretation focused on the importance of bilayer water interactions as the formation of the bilayer is caused by the hydrophobic effect. Definitely more experiments of these very complex systems are needed to test these suggestions for their validity.

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Registry No. LPC, 14863-27-5; DMPC, 13699-48-4; DPPC, 2644-64-6; DSPC, 4539-70-2; DAPC, 71259-34-2; DLPE, 42436-56-6; DMPE, 20255-95-2; DPPE, 3026-45-7; DSPE, 4537-76-2; DAPE, 87136-19-4; DMPA, 30170-00-4; DPPA, 19698-29-4; DTPA, 85763-37-7; DHPC, 18545-87-4; DHPE, 54285-60-8; DHPA, 23213-81-2.

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