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BIOLOGICAL STANDARD REFERENCE MATERIALS FOR THE CALIBRATION OF DIFFERENTIAL SCANNING CALORIMETERS: DI-ALKYLPHOSPHATIDYLCHOLINE IN WATER SUSPENSIONS

FREDERICK P. SCHWARZ

Chemical Thermodynamics Division, National Bureau of Standards, Gaithersburg, MD 20899 (U.S.A.)

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ABSTRACT

The temperatures and enthalpies of the phase transitions of suspensions of di-alkylphosphatidylcholines in buffered water solutions, prepared and stored under a variety of experimental conditions, were determined in a differential scanning calorimeter (DSC) to evaluate their potential as standard reference materials for the calibration of DSCs. The di-alkylphosphatidylcholine suspensions were 10 mass% 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine in aqueous buffered solutions at pH 7.0. A subtransition at 8.5 °C with an enthalpy of 15.5 kJ mol^{-1} was observed in the DMPC suspensions after storage of the sample at −5.5 °C for 2 days. The profile shape and the temperature and enthalpy values of the subtransition, the pretransition, and the ice peaks of the suspensions depended on the preparation and storage conditions of the samples. A relative standard deviation of 10% was obtained for the enthalpy values of the main transition and a standard deviation of 0.05 °C for the main transition temperature. Transition temperatures and enthalpies were also determined for suspensions of 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dieicosanoyl-*sn*-glycero-3-phosphocholine, and 1,2-di-9-*cis*-octadecenoyl-*sn*-glycero-3-phosphocholine in buffered water solutions.

INTRODUCTION

Differential scanning calorimeters (DSCs) are widely used in biological studies to determine the enthalpies and temperatures of conformational transitions occurring in proteins, nucleic acids, polysaccharides, and lipid assemblies in solution. The availability of DSCs capable of temperature measurements with precisions of ±0.1 °C and enthalpy measurements with precisions of ±10 μJ has created a need for temperature and enthalpy standards for calibration of the DSC measurements with a similar order of precision, particularly in the 0–100 °C temperature range. In addition, many of the high sensitivity DSCs used in biological laboratories contain fixed sample cells which are filled through capillary tubes, thereby limiting their usefulness to measurements on liquids. Calorimetric standards currently

available exhibit a solid-to-liquid phase transition and thus cannot be used with instruments requiring liquid samples. To minimize differences in thermal lag between the calibrations and measurements on biological systems, it would be preferable to use a calibration standard similar to the biological systems being studied. A solution of a lipid or protein exhibiting a well-characterized, reproducible enthalpy and temperature of transition would fulfil all of these requirements.

Suspensions of di-alkylphosphatidylcholines, a class of lipids commonly found in cell membranes, in buffered aqueous solutions have been suggested as standard reference materials for the calibration of DSCs used in biological studies. These suspensions exhibit sharp phase transitions with transition enthalpies on the order of 20–40 kJ mol^{-1} which are typical of the lower limit of enthalpies for phase transitions in biochemical systems such as proteins in solution. Recent reports [1,2], however, have suggested that the preparation and storage conditions of the suspensions will affect their thermotropic behavior and thus, perhaps, their performance as standard reference materials. Consequently, the reproducibility of temperature and enthalpy measurements of the phase transitions for a number of dialkylphosphatidylcholines has been determined for suspensions prepared and stored under a variety of experimental conditions typical of a biologically orientated laboratory.

EXPERIMENTAL

The phosphatidylcholines, 1,2-dimyristoyl-L-phosphatidylcholine (DMPC, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine), 1,2-dipalmitoyl-L-phosphatidylcholine (DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine), 1,2-distearoyl-L-phosphatidylcholine (DSPC, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine), 1,2-diarachioloyl-L-phosphatidylcholine (DAPC, 1,2-dieicosanoyl-*sn*-glycero-3-phosphocholine), and 1,2-dielaidoyl-L-phosphatidylcholine (DEPC, 1,2-di-9-*cis*-octadecenoyl-*sn*-glycero-3-phosphocholine) were obtained from Avanti Polar Lipids, Inc. with a stated purity of > 99 mol% and stored in a freezer. The lipids were analyzed by a gas liquid chromatography method [3]. Briefly, a sample of the lipid dissolved in ether was hydrolyzed by the addition of a methanol solution of sodium hydroxide and allowed to incubate at room temperature for 5 min. Microliter quanti-

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Certain commercial equipment, instruments and materials are identified in this paper in order to adequately specify the experimental procedure. In no case such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material, instruments or equipment identified is necessarily the best available for for the purpose.

ties of the ether solution were then injected into an HPLC equipped with a crosslinked methyl silicone gum capillary column operated at a starting temperature of 180°C with an increase of 5°C h^{-1}. The detector areas of the peaks of the alkyl acids resulting from the hydrolysis indicated a purity of greater than 99.8% by mass of the lipids DMPC, DPPC, DSPC, and DAPC. The DEPC was not analyzed.

Suspensions of the lipids in water were prepared by mixing weighed amounts of the lipid with weighed aliquots of 0.02 M sodium phosphate buffer (pH = 7.0), heating the mixture to a temperature several degrees above the main transition temperature of the lipid, and shaking the mixture at this temperature in a vortex mixer. The error in the weighing of the lipids and the buffer aliquots was estimated to be less than 0.1%. The pH of the buffered solution was measured using an Orion pH meter. The aqueous suspensions were then stored in an incubator at 1.5°C (slightly above freezing) or in a freezer at −35°C. Samples from the freezer were allowed to warm up to 1.5°C prior to transfer to the DSC cells. Approximately 0.4-ml samples of the buffered, aqueous suspensions were introduced into the DSC cells which had been previously equilibrated at 1.5°C. A cool pipette was used for the transfer to minimize any warming up of the suspensions. Temperature profiles resulting from thermal scans of the lipid suspensions immediately following transfer of the suspensions into the DSC cells were compared with those of suspensions that had been stored in the DSC at 1.5°C for the same period of time. The temperature profiles from the two measurements were indistinguishable, indicating that the transfer procedure did not affect the thermal properties of the suspensions. The loadings of the DSC cells were determined by weighing the cells after completion of the thermal scans. Finally, some of the solutions were prepared directly by weight in the cell. The cell was heated in the DSC to a temperature several degrees above the main gel-to-liquid crystal phase transition, and then cooled down to the lowest temperature of the thermal scan (1.5 or −5.5°C) for at least 1 h before the scan. All the suspensions were approximately 1.0 mass% in concentration and the DSC cells contained approximately 0.4 g of the lipid suspension.

The measurements were performed with a Hart 7707 DSC which is a heat flow scanning microcalorimeter consisting of two matched pairs of removable cells. Two samples were scanned simultaneously with each sample cell run against a reference cell containing an equal mass of the buffer solution. A heater located within the compartment of each cell was used to calibrate the thermopile response as a function of energy input. Calibration constants were determined every 10°C from 0 to 100°C and fit by the method of least squares to a second-order polynomial in temperature. The polynomial was then used to calculate the calibration constant for temperatures between 0 and 100°C. The voltage across the calibration heater was measured with a digital voltmeter calibrated with an NBS standard cell. The current of the

calibration heater was determined from a measurement of the voltage across a calibrated resistor connected in series with the calibration heater. All the calibration constants determined on empty cells agreed, within experimental error, with the calibration constants determined when the sample cells contained 1 ml of water. The temperature calibrations were performed at the factory and were checked against the known melting points of ortho-terphenyl and diphenyl ether. The diphenyl ether melts at 27.0°C with a heat of fusion of 17.2 kJ mol^{-1} [4] and the ortho-terphenyl melts at 56.2°C with a heat of fusion of 17.19 kJ mol^{-1} [5]. In thermal scans of five different samples with the DSC, the diphenyl ether was observed to melt at 27.3 ± 0.1°C with a heat of fusion of 17.06 ± 0.04 kJ mol^{-1} and the ortho-terphenyl to melt at 56.3 ± 0.1°C with a heat of fusion of 17.20 ± 0.07 kJ mol^{-1}. All the error limits are standard deviations determined from the average of the determinations. The largest standard deviation in the enthalpy measurements is 0.07 kJ mol^{-1} or, on a percentage basis, 0.4%.

The Hart DSC was operated at a scanning rate of 30°C h^{-1} except near the transition peaks where the scan rate was slowed to 5°C h^{-1} to minimize the effects of the calorimeter response time on the shape of the transition peaks. The response time was determined by turning off a calibration heater supplying 1 mJ of heat to a water-filled DSC cell and monitoring the decay of the millijoule signal as a function of time. The response time, which is defined as the time interval following a pulse of 1 mJ of heat into a water-filled cell to decay to 36.8% of its initial value, was approximately 2.5

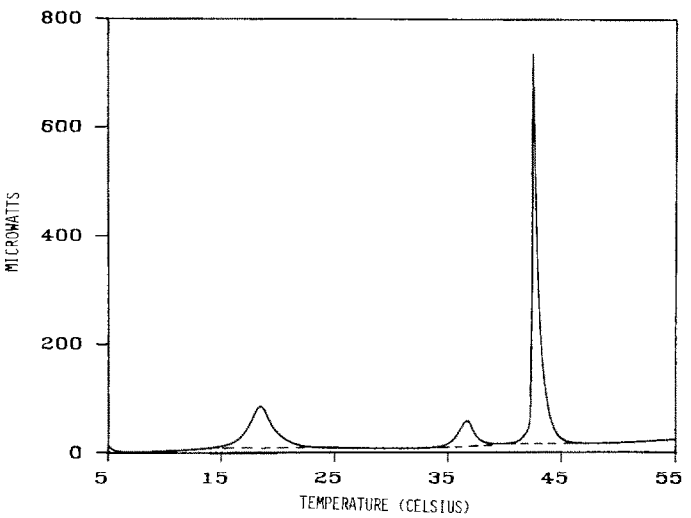

Fig. 1. Thermal scan of a 10% suspension of DPPC in 0.02 M sodium phosphate buffer (pH = 7.0) (Run 2 in Table 1) stored at 35°C for 14 days. The broken lines are the extrapolated baselines.

min. In the determination of an enthalpy value, the baseline of the transition peak was estimated by a straight line drawn between a lower temperature limit and an upper temperature limit as shown, for example, by the broken lines in Fig. 1. The lower temperature limit is where the thermal profile of the transition peak departs from the pretransitional baseline and the upper temperature limit is where the thermal profile returns to the posttransitional baseline.

RESULTS AND DISCUSSION

Typical phase transitions of a lipid–water suspension (DPPC in this case) are shown in Fig. 1 and consist of the following:

(1) The main gel-to-liquid crystal phase transition at 42.5°C. This phase transition is characterized by a disordering of the hydrocarbon chains from an all-*trans* configuration to a mixture of *trans* and *gauche* configurations [6].

(2) The pretransition in DPPC at 36.8°C. This transition has been characterized as a structural transformation from a one-dimensional (flat) lamellar structure to a two-dimensional lattice consisting of lipids distorted by a periodic ripple and increased disorder. The hydrocarbon chains which are tilted with respect to the normal to the bilayer axis at temperatures below the pretransition show a gradual decrease in the angle of tilt with increasing temperature and become parallel to the bilayer normal in the ripple phase above the pretransition [6].

(3) A subtransition peak at 18°C corresponding to a change in the hydrocarbon chain packing from orthorhombic to quasihexagonal. The subtransition is also believed to accompany the uptake of water in the headgroup region [7,8].

Transition temperature and enthalpy measurements for DPPC and DMPC are presented in Tables 1 and 2. The various experimental conditions for the preparation and storage of the suspensions are typical of those conditions reported in the literature by various laboratories [1,2,9–12]. The relative standard deviation of the mean value of ten determinations of the enthalpy of the main transition peak by DPPC is 9% and the corresponding standard deviation of the mean value of the temperature maximum is ± 0.05°C. The relative standard deviation of the mean value of six determinations of the enthalpy of the main transition peak of DMPC is 10% and the corresponding standard deviation of the mean value of the temperature maximum is ± 0.05°C. If the high and low enthalpy values are discarded, the relative standard deviation of the mean enthalpy values is reduced to 4% for the DPPC and the DMPC samples. This relative standard deviation is an order of magnitude greater than the relative standard deviations of the diphenyl ether and ortho-terphenyl enthalpy values and therefore reflects a variation

TABLE 1

Temperature maxima and transition enthalpies of the phase transitions of DPPC in 0.02 M sodium phosphate buffer suspensions (pH = 7.0)

Run No.	Sample preparation	Results: temperature in °C (enthalpy in kJ mol^{-1})			
		Ice peak	Subtrans. peak	Pretrans. peak	Main peak
1	stored at −35°C for 12 h			35.29±0.05 (2.10±0.06)	42.14±0.05 (32.9 ±0.3)
2	stored at −35°C for 14 days		13.48±0.05 (14.2 ±0.5)	35.77±0.05 (6.9 ±0.2)	42.14±0.05 (46.9 ±0.5)
3	stored at 1.5°C for 4.5 days		17.73±0.05 (16.3 ±0.5)	35.26±0.05 (4.4 ±0.1)	42.12±0.05 (37.8 ±0.4)
4	stored at 1.5°C for 10 days		7 ±0.05, 20.6±0.05 (double peak)	35.80±0.05 (6.32±0.03)	42.11±0.05 (38.9 ±0.01)
5	stored at 1.5°C for 17 days		20.20±0.05 (10.0 ±0.3)	35.67±0.05 (4.6 ±0.2)	42.11±0.05 (37.2 ±0.6)
6	stored at 1.5°C for 32 days		20.60±0.05 (14.7 ±0.2)	35.83±0.05 (5.5 ±0.3)	42.09±0.05 (36.6 ±0.5)
7	stored at 1.5°C for 40 days		20.69±0.05 (21.2 ±4.6)	35.80±0.05 (2.6 ±0.5)	42.06±0.05 (40.8 ±0.4)
8	fresh solution made up in cell w/o mixing, cooled to 1.5°C			35.56±0.05 (3.7 ±0.7)	42.12±0.05 (39.0 ±1.2)
9	same solution with mixing at 50°C, cooled down to 1.5°C			35.15±0.05 (2.4 ±0.2)	42.08±0.05 (40.9 ±0.4)
10	stored in DSC at −5.5°C for 3 days	2.79±0.05 (8.9 ±0.3) [a]	14.81±0.05 (11.7 ±0.3)	35.81±0.05 (3.81±0.04)	42.04±0.05 (38.1 ±0.4)

[a] Calculated in J mol^{-1} of water.

TABLE 2

Temperature maxima and transition enthalpies of the phase transitions of DMPC in 0.02 M sodium phosphate buffer suspensions (pH = 7.0)

Run No.	Sample preparation	Results: temperature in °C (enthalpy in kJ mol^{-1})			
		Ice peak	Subtrans. peak	Pretrans. peak	Main peak
1	stored at 1.5°C for 14 days			15.38 ± 0.05 (6.6 ± 1.3)	24.57 ± 0.05 (26.7 ± 0.3)
2	stored in DSC at −5.5°C for 2 h		7.38 ± 0.05 (4.4 ± 0.1)	15.37 ± 0.05 (2.72 ± 0.08)	24.56 ± 0.05 (22.2 ± 0.2)
3	stored at −5.5°C in DSC for 2 days		8.36 ± 0.05 (15.5 ± 0.5)	15.22 ± 0.05 (2.43 ± 0.07)	24.56 ± 0.05 (24.3 ± 0.2)
4	from run 3 stored in DSC at 1.5°C for 2 h			15.86 ± 0.05 (1.97 ± 0.06)	24.56 ± 0.05 (24.6 ± 0.3)
5	stored in DSC at −5.5°C for 5 days	2.57 ± 0.05 (5.79 ± 0.17) [a]	8.45 ± 0.05 (12.1 ± 0.4)	15.64, 17.42 (double peak)	24.58 ± 0.05 (22.3 ± 0.2)
6	stored in DSC at −5.5°C for 3 days	2.69 ± 0.05 (5.97 ± 0.18) [a]	8.53 ± 0.05 (10.5 ± 0.3)	15.30, 1744 (double peak)	24.52 ± 0.05 (24.2 ± 0.2)

[a] Calculated per mole of water.

in the exhibited thermal properties of the samples. It should also be noted that the largest deviations from the mean DPPC enthalpy values arose from those samples that were transferred from the freezer to the DSC cells as opposed to those samples stored in the DSC at 1.5°C. As shown in Table 3, similar standard deviations were obtained for the enthalpies and temperatures of the main transitions of the other lipids studied.

The transition peak in the DMPC samples near 8°C depicted in Table 2 and in Fig. 2b has not been reported in the literature and is tentatively identified here as a subtransition peak. The subtransition in DPPC, however, has been well characterized. Upon incubation of a DPPC suspension at low temperatures such as −2.0°C, the bilayer is reported to dehydrate slowly in the headgroup region from 19 mol H_2O/mol DPPC to 11 mol H_2O/mol DPPC and to assume a more rigid orthorhombic configuration [10]. Therefore the enthalpy of the DPPC subtransition, which is the reverse of this process, increases slowly with storage time, with a maximum value observed after 3 days of incubation at 0°C [1]. In addition, the temperature of the DPPC subtransition increases with storage time and the DPPC samples must be stored for at least 6 h at temperatures well below the subtransition temperature for re-appearance of the subtransition peak in the samples [1]. More specifically, the DPPC subtransition gradually shifts from a single peak at 16.3°C with a shoulder at 18.7°C to a single peak at 20.7°C after incubation at 1.5°C for 17 days (Fig. 3). Similar behavior is also observed with the DSPC subtransition [1]. As shown in Table 2, the DMPC samples must be stored at 5.5°C for the subtransition to appear and the maximum

TABLE 3

Phase transition temperatures and enthalpies of the lipids in buffer solution

Lipid	Experimental values Temp. (°C)	Enthalpy (kJ mol^{-1})	Literature values		Ref.
			Temp. (°C)	Enthalpy (kJ mol^{-1})	
DMPC Main trans.	24.56±0.05	23.9 ±2.0	23.70±0.09	26.2±0.7	2
			23	27.8	15
Pretrans	15.5 ±0.3	3.4 ±2.0	13.5 ±0.2	4.6±0.9	2
Subtrans	8.0 ±0.6	13.1 ±0.6			
DPPC Main trans.	42.10±0.05	38.3 ±1.6	41.75±0.06	40.6±0.8	2
			41	36.3	15
Pretrans	35.5 ±0.3	4.25±1.64	34.0 ±0.2	9.6±0.8	2
Subtrans.	17 ±3	21.3 ±0.8	19.8	23.4	10
DSPC Main trans.	55.36±0.05	44.0 ±2.1	58	45.2±0.8 [a]	2
				44.8	15
Pretrans.	51.6 ±0.1	4.6 ±0.4	49.1 ±0.2	5.9±0.4	2
Subtrans.	14–17	1.7 ±0.5			
DAPC Main trans.	65.53±0.05	62.4 ±0.8			
Pretrans.	63.2 ±0.2	2.9 ±0.4			
DEPC Main trans.	12.59±0.05	38.1 ±0.8			
Pretrans.	8.7 ±0.2	0.42±0.08			

[a] Reference 2 gives two values for the main transition of DSPC, one at 58.24°C and one at 54°C.

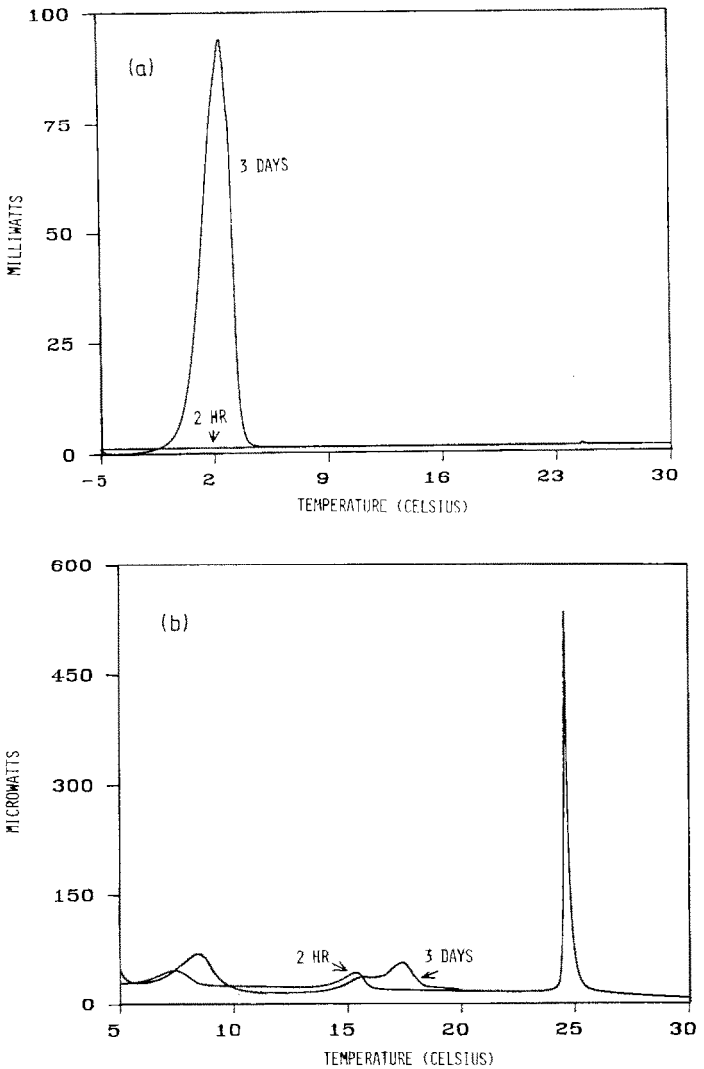

Fig. 2. Thermal scans of 10% suspension of DMPC in 0.02 M sodium phosphate buffer (pH = 7.0) stored at −5.5°C for 3 days and for 2 h. Scale (a) = 1000 × scale (b).

enthalpy of this transition (15.5 kJ mol^{-1}) is obtained only after incubating the sample at this temperature for 2 days. The transition temperature also increases by approximately 1°C with an increase in storage time from 2 h to 3 days at −5.5°C. Therefore, the transition appearing around 8°C is assumed to be the subtransition of the DMPC suspensions. Although the subtransition of DMPC has not been reported previously using calorimetric methods, Westerman et al. have detected by ^2H NMR measurements on DMPC suspensions a transition at −4°C which they termed the subtransition of DMPC [13]. The temperature difference between the calorimetrically

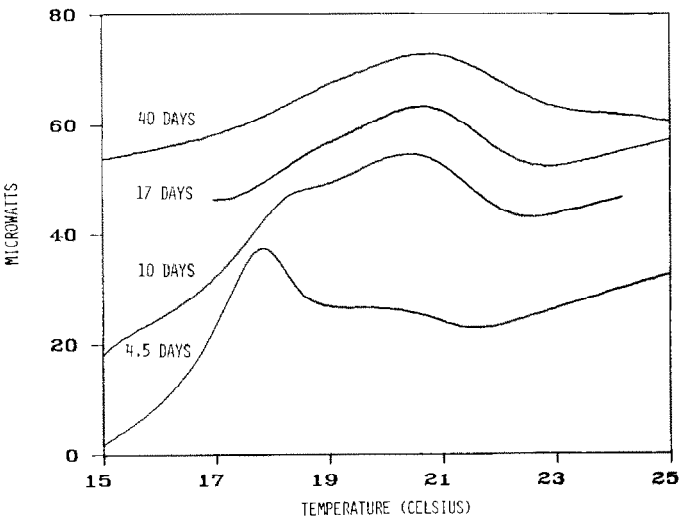

Fig. 3. Thermal scans of 10% suspension of DPPC in 0.02 M sodium phosphate buffer (pH = 7.0) stored at 5.5°C for 4.5, 10, 17, and 40 days.

observed subtransition and the NMR monitored subtransition could result from the difference in the sample preparation and storage conditions. The NMR samples contained only 58–72 mass% water and were stored at −80°C for a few days and then at −20°C for a few hours prior to the measurements.

The enthalpies and the temperatures of the pretransitions depend on prior treatment of the sample as in the case of the subtransitions. This is shown in Tables 1 and 2. The relative standard deviation of the mean enthalpy of the pretransition peak in the DPPC samples is 25% which is higher than the corresponding relative standard deviation of 9% for the main transition enthalpy value. Storage of the DMPC samples at −5.5°C results in the appearance of a second transition peak at a temperature approximately 2°C higher than the pretransition temperature and, thus, overlaps the pretransition peak. The DMPC and DPPC samples stored at −5.5°C also exhibit an ice peak at 2.7°C. The enthalpy of the ice peak transition in the DMPC samples (Fig. 2a) is, however, almost three orders of magnitude larger than that of the DPPC samples. The ice peak enthalpy values for the DMPC samples are close to that of pure water (6 kJ mol^{-1}) indicating the freezing of almost all the water in the sample. The appearance of a second peak at a temperature approximately 2°C higher than the pretransition peak maximum in the DMPC samples stored at −5.5°C may result from freezing of all the water in the sample. Kodama et al. [14] observed that in powdered DPPC samples containing small amounts of water (15–25 mass%), the pretransition peak appears simultaneously with an ice peak transition near 2°C. As the amount of water in the sample increases, the enthalpies of the

ice peak and of the pretransition peak increase while the pretransition peak temperature maximum decreases toward the pretransition temperature of the suspensions [14]. This behavior in DPPC is explained as the influence on the nature of the pretransition of the uptake of water in the headgroup region of the lipid bilayer, an explanation similar to that given for the behavior of the subtransition [14].

The transition enthalpies and transition temperatures determined in this study for the DSPC, DAPC, and DEPC suspensions are presented in Table 3 along with the DMPC and DPPC values. The values for DSPC, DMPC, and DPPC are close to the literature values [1,2,10,15]. The presence of a double bond in the alkyl chains of the DEPC causes a shift of the pre- and main transitions to lower temperatures when compared to its saturated form, DSPC. No subtransition peaks were observed for the DAPC and the DEPC samples. The subtransition peak in the DEPC samples may occur below the freezing point of water. A subtransition peak for the DAPC mixtures was not observed even after prolonged incubation of the mixtures up to 40 days at 1.5°C. In the DSPC samples, an additional peak not reported in Table 3 was observed between the pretransition peak and the main transition peak when the samples were rapidly cooled down or stored for a short time at a low temperature. Prolonged storage of the DSPC mixtures at low temperatures caused this peak to disappear. Similar behavior was also observed by Hinz and Sturtevant [2].

CONCLUSION

The enthalpies and temperatures of the pre- and subtransitions, both of which are not fully understood, depend on the conditions of preparation and storage of the samples. Moreover, the main transition peak temperature maximum had been observed to shift slowly from a lower temperature to the normal transition temperature when the sample is prepared by sonication instead of vortex mixing at temperatures above the main transition temperature [16,17]. Although the general features of the sub- and pretransitions can be reproduced, the enthalpies and temperatures of the temperature profiles cannot be reproduced to within the measurement capabilities of modern high-accuracy, high-sensitivity DCSs. Moreover, conditions suitable for the manifestion of reproducible calorimetric behavior of one lipid may not be appropriate for reproducible behavior in another lipid. Even the relative standard deviation of the main transition enthalpy values is on the order of 10% which, for the typical 10% lipid suspensions studied here and the 0.5 g DSC loadings, corresponds to a sensitivity of only about 20 mJ. In contrast, the main transition temperature maximum exhibits a low standard deviation on the order of ± 0.05°C for all the lipids studied which cover a temperature range from 12.59 (DEPC) to 65.53°C (DAPC). The lipid suspensions would

thus make good temperature standard reference materials for DSCs. It should be emphasized that the precision of the temperature measurements depends on maintaining a constant scan rate. In order to compare the temperature maxima from several laboratories, the scan rate of the calibrations should be specified or the temperature maxima could be determined at different scan rates and then extrapolated to zero scan rate. The temperature calibrations could then be defined in terms of zero scan rate. Furthermore, the suspensions should be made up on a vortex mixer and not sonicated, otherwise the main transition peak temperature will be shifted downward.

Since the water lipid suspensions do not appear to make reasonable standard reference materials for the calibration of DSCs, proteins, which undergo conformational changes in buffer solutions, are now being investigated as possible DSC standard reference materials. Preliminary measurements on buffered solutions of bovine pancreatic ribonuclease-a in water indicate that these solutions may make good standard reference materials for enthalpy calibrations of DSCs. Ribonuclease-a undergoes an unfolding transition in solution between 31.5 and 69°C [18] with a temperature maximum and an enthalpy value which depend on the pH of the solution. The preliminary measurements show that the enthalpy of transition increases monotonically with the transition temperature by a factor of almost 2 over this temperature range. The determination of the suitability of this protein or other proteins will depend upon the accuracy with which calorimetric behavior can be reproducibly related to solution parameters such as pH and electrolytic strength.

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