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Perturbation of the chain melting transition of DPPC by galactose, agarose and Laurdan as determined by differential scanning calorimetry

Susan B. Abrams and Paul Yager

Center for Bioengineering, FL-20, University of Washington, Seattle, WA (USA)

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Differential scanning calorimetry was used to determine the effects of agarose hydrogel, galactose and the fluorophore Laurdan on the thermal behavior of multilamellar liposomes of DPPC. Although the effect of agarose on the phase transition was found to be negligible at low concentrations ($\leq 2.5\%$), higher concentrations result in an endotherm that broadens and splits into two calorimetric events, one of which is at a higher temperature than that of hydrated DPPC. Equal weight fractions of galactose produce similar effects, although both fractions have raised melting temperatures. The higher melting components may be produced by osmotically-driven dehydration of the inner liposomal monolayers, a physical interaction between the carbohydrates and lipid headgroups, or a combination of both. Laurdan has little effect on the phase transition of DPPC vesicles at the concentration used in the sensor (0.67 mol%); concentrations up to 5.4 mol% only slightly lowered the melting temperature.

Introduction

The anesthetic potency of general anesthetics has been correlated with solubility in a lipid bilayer [1] and can be monitored via the resulting depression in lipid melting temperature. This effect has been used as the basis for development of an optical fiber sensor for in vivo monitoring of general anesthesia [2–7]. Large multilamellar vesicles (MLV) of dipalmitoylphosphatidylcholine (DPPC) are labeled with an order-sensitive fluorophore and immobilized at the tips of optical fibers by embedding in agarose hydrogel in order to monitor anesthetic levels in otherwise inaccessible locations.

While little is known about interactions of carbohydrate polymers such as agarose with phospholipids, mono- and disaccharides are known to be able to preserve membrane structure in adverse conditions such as freezing or dehydration (see Ref. 8 for review). High concentrations of such carbohydrates can substantially affect the gel-to-liquid crystalline phase transition in phospholipids by two distinct mechanisms. Characteristic chain melting temperatures (T_m) of phospholipid dispersions increase as the activity of water decreases in the presence of increased solutes [9–11], and there is also evidence that many sugars can stabilize bilayers by hydrogen bonding to lipids in place of water [10,12], most likely at the phosphodiester group in the lecithin head group [13,14]. Strauss et al. [10] found that addition of more than 10% sucrose to hydrated multilamellar vesicles of DPPC elevated the melting temperature; they suggested a hydrogen bonded sucrose network as the cause. Rudolph et al. [12] proposed that the interaction of carbohydrates with lipid headgroups causes an alteration in the packing density of a bilayer, so the effect is sensitive to the pre-existing packing; they noted that the amount of trehalose that raised the melting temperature in MLV lowered T_m in small unilamellar vesicles. Gruner et al. [15] found it unlikely that solutes in the wetting buffer are incorporated into the interior of MLV; these vesicles are under osmotic pressure that condenses the entire vesicle and dehydrates the interior bilayers. On the other hand, cycling through the phase transition temperature has been shown to reduce, if not eliminate, transbilayer concentration differences [16].

Crowe and Crowe [16] found that several mono- and disaccharides raise T_m and broaden the main transition of DPPC MLV. However, addition of sugars to uni-

Correspondence to: S.B. Abrams, Bioengineering, FL-20, University of Washington, Seattle, WA 98195, USA.

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MLV, multilamellar vesicles; SUV, small unilamellar vesicles; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; FWHM, full width at half maximum.

lamellar vesicles created multiple thermodynamic populations. High concentrations of trehalose, sucrose, and fructose created a low-temperature shoulder on the DPPC endotherm, indicating a second population with a lower T_m than that of pure hydrated DPPC. Glucose at low levels lowered T_m but at higher levels caused a shoulder to appear on the high-temperature side of the endotherm, the relative size of which increased with sugar concentration. They suggest that with all of these sugars, the lower transition is caused by some sort of binding that increases headgroup spacing, and the higher-temperature transition is produced through dehydration of some portion of the lipids. These studies of small sugars are not yet definitive, and we are aware of no comparable studies of the thermal effects of high molecular weight polysaccharides.

The membrane-soluble fluorescent probe 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan) has been used to monitor the phase transition optically [17] and allows convenient two-wavelength ratiometric quantification of lipid 'fluidity' [3]. Most fluorescent probes will, at even modest concentration, affect the phase transitions of lipid bilayers in which they are present because of preferential solubility in one phase or the other. Fumero et al. [18] explored the effects of several fluorescent bilayer probes on the thermal behavior of L-α-dimyristoylphosphocholine multilamellar suspensions. Little effect was seen when the dyes were used at concentrations up to 4 mol%. At 5 mol%, there were changes in the enthalpy of the transition but no effect on the melting temperature. Sensor optimization requires use of Laurdan at a level that will not alter the phase transition.

This paper describes differential scanning calorimetry measurements to determine the effect, if any, of Laurdan and agarose on the chain melting phase transition of multilamellar DPPC vesicles. Agarose is primarily composed of D- and L-galactose residues, so to attempt to differentiate osmotic effects from those of direct molecular interactions, the effects of galactose on T_m have also been studied.

Materials and Methods

Lipids and other reagents

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, M_r 734.05) in chloroform was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification (purity > 99%). The fluorescent dye Laurdan (6-lauroyl-2-(dimethylamino) naphthalene, M_r 353.55) was obtained in powder form from Molecular Probes (Eugene, OR) and dissolved in chloroform at concentrations ranging from 0.1 to 0.3 mg/ml several hours before use. Agarose (mol. biol. reagent No. A-0169, type I-A, Low EEO, < 0.35% sulfate), D-(+)-galactose (mol. biol. reagent No. G-0625), and L-galactose (mol. biol. reagent No. G-7134) were purchased from Sigma (St. Louis, MO). In all studies, the buffer contained 10 mM Hepes, 100 mM NaCl (pH 7.5).

Lipid / fluorophore combination

Fluorophore and lipid were first mixed in chloroform in a 100 ml round-bottom flask, usually at a ratio of 1 dye molecule per 150 lipids. Solvent was removed by rotary evaporation at 50°C followed by evacuation for 8 h. The lipid-dye mixture was then rehydrated with aqueous buffer (10 mM Hepes, 100 mM NaCl in distilled deionized water (pH 7.5)) and dissolved by warming to achieve a lipid concentration of approx. 100 mg/ml. These samples were later diluted with buffer or sugar solutions to produce a final lipid concentration of 75 mg/ml.

Vesicle preparation

Large multilamellar vesicles were formed by vortexing at a temperature above the T_m of aqueous DPPC multilamellar dispersions (41.4°C) [19] for a total of at least 10 min. To do this, a small volume of aqueous lipid suspension (75 or 100 mg lipid/ml) was heated to approx. 55°C in a sealed test tube. The heated mixture was vortexed (Vortex Genie Mixer S8223, Scientific Products, Evanston, IL) until the temperature recorded by a thermocouple inside the test tube dropped to 42°C, which usually occurred within 20 to 40 s. The sample was then reheated and this procedure repeated until at least 10 min of total vortexing had elapsed. The majority of the resulting lipid aggregates range in diameter from 0.4 to 10 μm.

Vesicle / carbohydrate combination

In most studies, agarose or galactose powder was hydrated with warm buffer to a concentration ranging from 4–30%. The carbohydrate solution was heated to above the agarose gelling temperature (36°C), stirred for 5–6 min, and then mixed with three parts of heated MLV lipid/fluorophore suspension. All components were maintained above the lipid phase transition temperature throughout the process. The resulting lipid concentration was 75 mg/ml and carbohydrate concentrations ranged from 1% to 7.5%. Controls were prepared by mixing lipid with warmed buffer. In the first galactose experiment, a racemic mixture of L-galactose and D-(+)-galactose was used as a model for agarose subunits. Subsequent studies used D-galactose. In several experiments, the carbohydrate was added to the lipid sample and thoroughly dissolved at a temperature above T_m of the lipid before vesicle formation.

Differential scanning calorimetry

Differential scanning calorimetry was performed using a Seiko Instruments DSC100 differential heat flux

microcalorimeter. Silver sample pans (nominally 15 μl volume) were employed, the bottoms of which were smoothed with lapping film to increase thermal contact with the pedestal. Reference and sample pan masses were matched to within 6.2% total mass always, and usually to within 2.5%. A two-point temperature calibration of the calorimeter was performed using indium (T_m = 156.6°C) and gallium (T_m = 29.8°C) in silver pans. The one-point enthalpy calibration suggested by Seiko was performed with gallium but appeared to be unreliable. Small changes in protocol and equipment necessitated periodic recalibration of the system over the course of these experiments and may obviate direct comparison between groups of results.

DSC operation near room temperature requires the use of a subambient heat sink. Initially, nitrogen gas flowing through copper tubing submerged in liquid nitrogen was applied to the heating block in the DSC. In subsequent studies, a coil of copper tubing was submerged in an ethanol bath and the head of a Cryocool CC-65A immersion cooler (Neslab Instruments, Newington, NH) was inserted into the bath without contacting the copper tubing. A gas flow of 4 to 6 l/min provided a strong enough differential to maintain the linearity of the temperature ramp between 20°C and 60°C.

Small DSC pans were loaded with up to 20 μl of lipid suspension, resulting in 1.0–1.5 mg lipid in each pan. The reference cell contained a matching volume of buffer. Prepared pans were hermetically sealed with silver lids and stored in the refrigerator until use. The temperature was scanned at 0.2 C°/min, data were sampled every 1 to 2 s, and data from the first scan were discarded to avoid mixing artifacts.

Because of recalibrations and changes to the cooling system, a control sample, containing the basic lipid preparation (75 mg/ml with 0.67 mol% Laurdan) without carbohydrate, accompanied each set of studies. The endotherm generated during the second scan of this control sample was used as a reference template for analysis of the accompanying studies, as discussed below.

Initially, a range of agarose concentrations was tested (0%, 2.5%, 5.0%, 7.5%) by mixing the same vesicle suspension with different polysaccharide solutions. The effect of 5.0% galactose was tested for comparison with the agarose system. In later studies, the carbohydrates were added to the lipid sample before vesicle formation to determine the extent of the effect of the association with lipid headgroups. In all carbohydrate studies, lipid samples also contained fluorophore at a concentration of 0.67 mol% (1:150). To explore the effect of the fluorophore Laurdan on the DPPC chain melting transition, the concentration of dye in the absence of sugars was increased to 2.6 mol% (1:38) and 5.4 mol% (1:18).

Analysis

Seiko DSC system software allowed determination of four types of data from each endotherm: the extrapolated onset temperature of transition, T_m, the temperature at the peak of the transition, the full width at half maximum (FWHM) of the endotherm, and the change in enthalpy with heating. Because of inadequate calibration, enthalpy data are not reported here. Peak transition temperature and FWHM were inappropriate metrics of broadened endotherms; therefore, further analysis was performed on a Macintosh II personal computer using Igor® Graphing and Data Analysis Software from Wavemetrics (Eugene, OR). In addition to multiple peak curve fitting, this software permitted subtraction of y-offset and correction for time-based drift. Endotherms from DPPC MLV with 0.67 mol% Laurdan (no carbohydrate) served as controls for each group of experiments. Because the asymmetric control endotherms were not well modeled by standard statistical distribution functions, such as the Gaussian or Lorentzian, an alternate modeling template had to be developed in order to derive separate information from the two populations seen in some of our thermal spectra. Each control endotherm was fit to the product of two exponential sigmoids of opposite polarity and then normalized. The resulting sequences of points were used as templates for data scans performed under the same cooling and calibration conditions by varying the magnitude and width of the template and summing as many components as were deemed necessary by visual inspection. Because of a consistent depression of the leading baseline in the data files, this region was de-emphasized during the modeling by excluding a small leading baseline section. The peak value coordinates and FWHM of each fit component were compared, and ratio of the areas of the two components is assumed to represent the relative sizes of populations in each sample.

Results

Control studies

The experiments presented in this report were performed in five groups. The control scans for each of these groups varied slightly, depending on the state of the cooling system and the calibration. Results of analysis of the control scans are listed in Table I. Based on eight control scans (some not reported here), the mean transition onset temperature was 41.22°C with a standard deviation of 0.02 C°. The mean full width at half maximum of the five controls presented here was 0.22°C (±0.03 C°). Since analysis of results between subsets of scans is invalid, data are presented as shifts or differences from the control studies.

TABLE I

Characteristic features of endotherms derived from 'control' scans

DSC endotherms were generated by scanning samples of DPPC MLV (75 mg/ml lipid) containing 0.67 mol% Laurdan at 0.2°C/min. In all cases, results were derived from the second heating scan. Onset T_m is based on the unprocessed endotherm. Reported peak T_m is from the smoothed version. FWHM is reported for both the unprocessed and the smoothed versions of the data. Dotted lines indicate changes in instrumentation or calibration. The five scans shown here served as controls for the data presented in this paper.

Scan	Onset T_m (°C) (raw)	Peak T_m (°C) (smoothed)	FWHM (C°) (raw)	FWHM (C°) (smoothed)
1	41.24	41.35	0.19	0.20
2	41.19	41.40	0.26	0.28
3	41.14	41.28	0.23	0.23
4	41.09	41.25	0.22	0.22
5	41.10	41.23	0.21	0.20

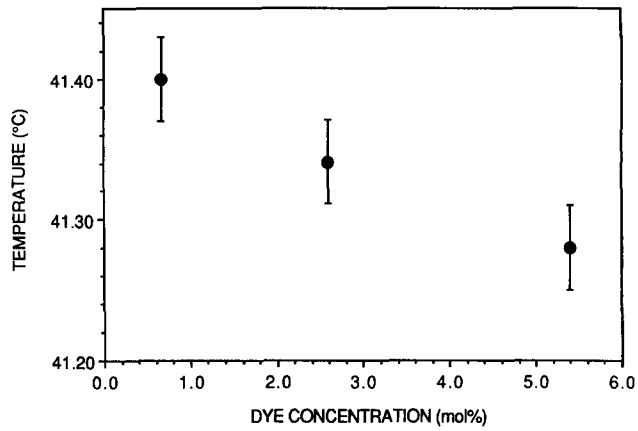

Fig. 2. DPPC MLV phase transition temperature decreases with increasing Laurdan concentration. Each point represents the center of a single endotherm, as determined after modeling. Error bars are estimated from repeatability of control scans.

Lipid / dye interaction studies

To determine the effect of the addition of Laurdan on the thermal characteristics of DPPC, three fluorophore concentrations were tested, ranging from the control level (0.67 mol%) to 5.4 mol%. The endotherms obtained in the DSC for each of the preparations have similar shapes and are shown in Fig. 1. Although no endotherms are reported here for DPPC MLV in the absence of fluorophore, studies of this preparation under slightly different conditions suggest no substantial difference from the controls in this report. Because there is no obvious evidence of a second melting species and the change in shape of the endotherm for higher dye concentrations is slight, modeling results are not reported. Attempts to model these data revealed a subtle but consistent change in shape with increasing fluorophore; the peak of the endotherm becomes sharper and the high-temperature trailing edge drops off more slowly than in the control files. Only for the highest concentration (5.4 mol%) is the change in transition temperature statistically significant, although there appears to be a general downward trend in phase transition temperature with dye concentration (Fig. 2).

Lipid / carbohydrate interaction studies

Increasing concentrations of agarose (0%, 2.5%, 5.0%, 7.5%) broaden the melting endotherm by producing what appear to be two separate populations, as shown in Figs. 3 and 4. The results of analysis of these studies are shown in the upper rows of Table II. Data from a later scan with 1% agarose, the level used in our sensor, are also included; note that addition of 1% agarose did not broaden the endotherm enough to justify modeling it as the sum of two components. The increase in the overall FWHM becomes statistically significant at the 5% and 7.5% agarose levels, as is

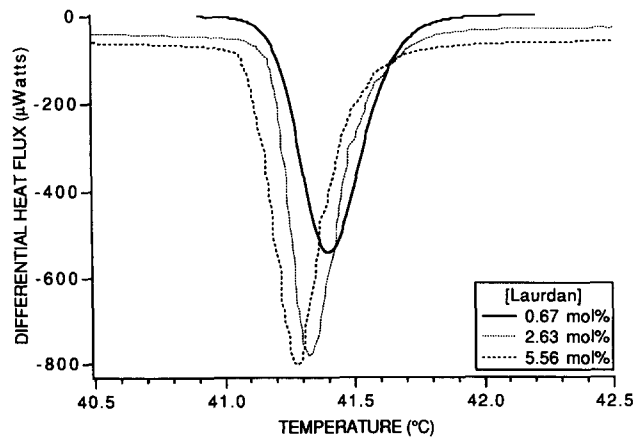

Fig. 1. Calorimetric endotherms produced by increasing the concentration of the fluorophore, Laurdan. Spectra have been corrected for small baseline slopes and they are offset here for display purposes.

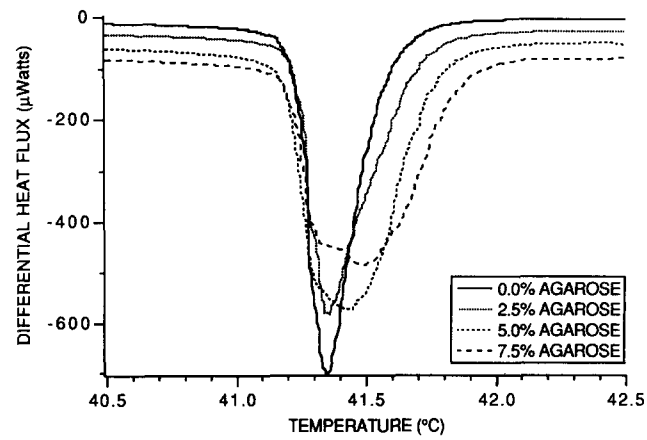

Fig. 3. Calorimetric endotherms produced by increasing the concentration of agarose added to DPPC MLV. Processing for modeling analysis includes correction of slight baseline slopes and subtraction of y-offsets. The spectra shown here have been processed and are offset from each other for display purposes.

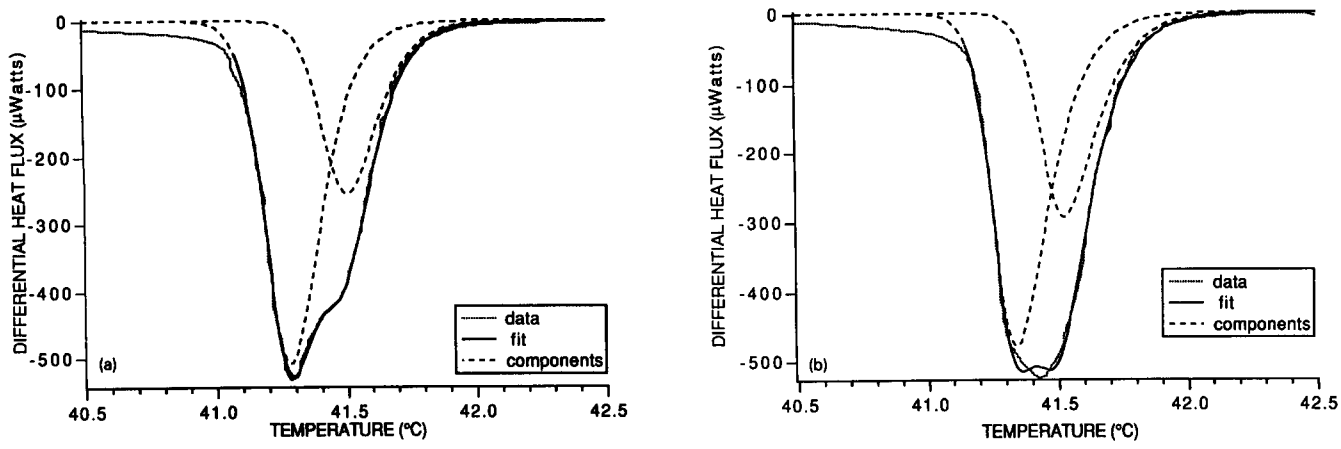

Fig. 4. Examples of 2-component modeling that uses templates from control DPPC MLV scans. (a) 5% agarose was added to the lipid/dye suspension before vesicles were formed. The 2-component fit (line) matches the data points well. (b) Another example of the effect of 5% agarose, this time added after vesicle formation. Note that the difference in shape between A and B is representative of reproducibility problems between groups of experiments and is not a function of sugar addition or vesicle formation sequence.

visible in Fig. 5. The transition onset temperature, which reflects the position of the low temperature peak, does not appear to change with the addition of agarose. The areas of the fit components were compared by examining the proportion of the area of the higher-temperature component in the total endotherm area. Note that the size of the second component somewhat parallels the agarose content (Fig. 6).

A similar experiment with 5% of a racemic mixture of D-(+)-galactose and L-galactose was performed twice. In both scans, the endotherms were broadened even more than with 5% agarose scan (Fig. 6, upper galactose points). Although galactose also produces two populations, the separation between them appears to be greater than in agarose and both components melt at significantly higher temperatures than the control with no carbohydrate (Fig. 7). The lower part of Table II summarizes the data from the combination of preformed DPPC/fluorophore vesicles with 5% galactose.

Another set of studies compared the effect of adding carbohydrates before and after vesicle formation. The three control files from these studies (DPPC MLV with 0.67 mol% Laurdan and no carbohydrate) were performed under the same instrumental conditions and indicate a mean transition temperature of 41.11 ± 0.03°C. The results in Table III again show that both monomeric galactose and polymeric agarose split the DPPC endotherm into two melting species. Although addition of the carbohydrate before vesicle formation appears to produce some qualitative differences in the shape of the endotherm (not shown), these differences

TABLE II

The effect of carbohydrate concentration on the phase transition of DPPC MLV

MLV suspensions containing 75 mg/ml DPPC and 0.67 mol% Laurdan were combined with increasing concentrations of agarose or equimolar D- and L-galactose. Samples were scanned at 0.2 C°/min. In all cases, the second scan endotherm was used. Each row represents a single scan. All numbers are indicative of differences either from control scans or between components. Respective errors in T_m, FWHM, and proportional area are 0.02 C°, 0.03 C° and 6% (agarose) or 4% (galactose).

Carbohydrate	%	Difference from control				Difference between components	% Area in comp 2
		raw endotherm		modeled components			
		onset T_m (C°)	FWHM (C°)	peak T_m (C°) of comp 1	peak T_m (C°) of comp 2	peak T_m (C°)	
Agarose	1.0	+0.03	+0.06	+0.07	–	–	–
Agarose	2.5	−0.01	+0.06	+0.01	0.19	0.18	16
Agarose	5.0	−0.06	+0.21	−0.01	0.17	0.18	38
Agarose	7.5	+0.01	+0.21	−0.08	0.14	0.22	74
Galactose	5.0	+0.13	+0.29	+0.12	0.43	0.31	45
Galactose	5.0	+0.22	+0.35	+0.23	0.57	0.34	41

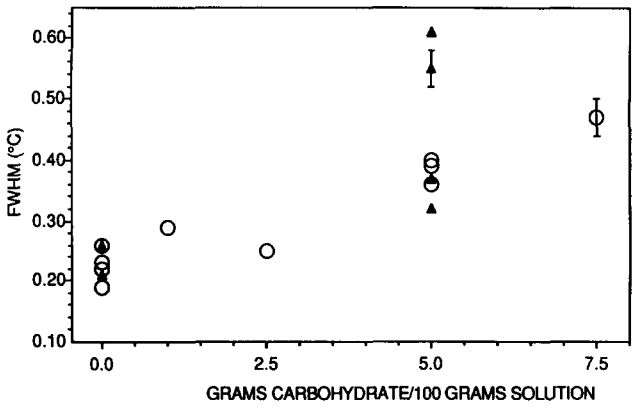

Fig. 5. Full width at half maximum height increases with addition of agarose or galactose. Open circles represent agarose studies or controls that accompanied agarose studies. Filled triangles represent galactose studies and the accompanying controls. All symbols represent a single scan. Error bars are ±0.03 C° as shown and are based on reproducibility of control files.

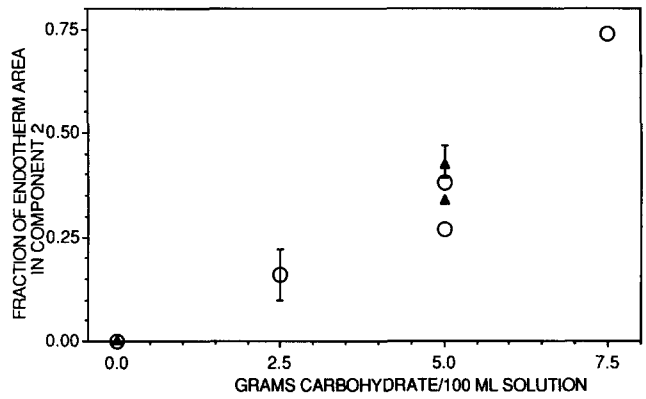

Fig. 6. Fractional area of the endotherm occupied by the high-temperature component increases with weight% carbohydrate. Open circles indicate agarose results and each represent a single scan. Closed triangles represent galactose studies, one of which is an average of two scans performed under the same conditions. Representative error bars are shown and are based on the reproducibility of three scans at the 5% level for each saccharide.

are not apparent in the onset temperature, the FWHM, or the distribution of endothermic area between the two components in the case of agarose. The addition of 5% D-galactose increased the melting onset temperature of the entire endotherm by 0.11°C for both preparation sequences. However, addition of galactose before vesicle formation resulted in a smaller high-temperature component than when added to preformed vesicles.

Some of the studies in Table III duplicated those reported in Table II, yet there are some differences in the results. One difference is manifested in the proportional size of the second peak, which is smaller for both carbohydrates in this second set of studies. Another difference is in the temperature separation between the two galactose peaks, which appeared quite large (approx. 0.32 C°) in the earlier studies but is more similar to agarose for the studies reported in Table III (approx. 0.22 C°). Fig. 3 is representative of the qualitative differences seen between each set of studies. The widths of the individual components are comparable in all the carbohydrate fits except in the 7.5% agarose scan, in which the high-temperature peak is significantly wider and the low-temperature peak slightly narrower. Since the modeled widths of the components are highly dependent on the aspect ratio of the template, slight deviations in template shape could affect the results obtained using this fitting technique.

Modeling technique

Attempts to fit unimodal endotherms to analytical functions (e.g., Gaussian, Lorentzian) were unsuccessful. The transfer function of the Seiko microcalorimeter causes 'Gaussian events' to be reported as asymmetrical peaks with a steep low-temperature edge and a shallower trailing edge (see Figs. 3, 4 and 6). The

TABLE III

The effect of the presence of carbohydrate during vesicle formation on the phase transition of DPPC MLV

In all cases, 5% sugar was added to the lipid/dye suspension to produce a final lipid concentration of 75 mg/ml and Laurdan at 0.67 mol%. Samples were vortexed at a temperature above T_m for a total of 10 min either before or after addition of carbohydrate. The galactose in these studies was of the D-form. All numbers represent differences from controls or between components. Each row is based on a single scan. Respective errors in T_m, FWHM, and proportional area are 0.02 C°, 0.03 C°, and 6% (agarose) or 4% (galactose).

Carbohydrate	Preparation sequence	Difference from control				Difference between components	% area in comp 2
		raw endotherm		modeled components			
		onset T_m (C°)	FWHM (C°)	peak T_m (C°) of comp 1	peak T_m (C°) of comp 2	peak T_m (C°)	
Agarose	vortex-sugar	+0.02	+0.14	+0.05	0.30	0.26	26
Agarose	sugar-vortex	+0.01	+0.17	+0.07	0.28	0.22	26
Galactose	vortex-sugar	+0.10	+0.16	+0.11	0.27	0.16	34
Galactose	sugar-vortex	+0.14	+0.11	+0.11	0.29	0.18	20

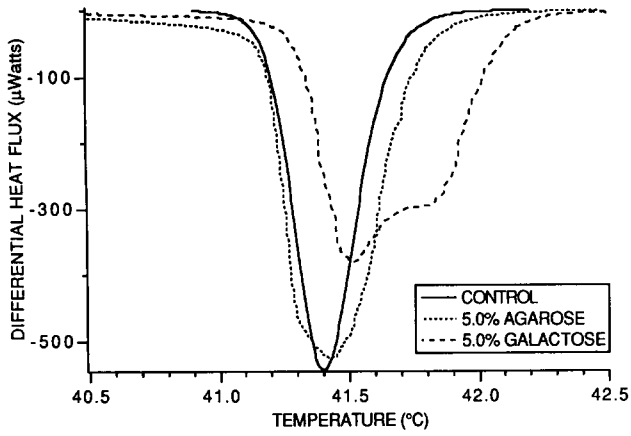

Fig. 7. Examples of calorimetric endotherms for a control file, a 5% agarose sample, and a 5% galactose sample. In all cases, carbohydrate was added to preformed vesicles. *y*-offsets have been removed and spectra have been corrected for small baseline slopes.

Gaussian function modeled accurately the leading edge of the endotherm but deviated significantly on the high-temperature side. On the other hand, a Lorentzian function fit the trailing high-temperature edge quite well but deviated on the low-temperature side. The method of fitting bimodal data with a normalized template of control data produced satisfactory results in most cases; the best and worst examples are shown in Fig. 4.

The technique was adequate for evaluation of the carbohydrate studies, but changes caused by high levels of fluorophore were not well characterized. The only region of the fit that was consistently poor in the carbohydrate studies was in the baseline region on the low temperature side, where the slight depression may be attributed to residual enthalpy from the lipid pretransition. Full analysis of the transfer function of the Seiko DSC 100 and its effects on calorimetric data is beyond the scope of this project. Some minor problems in the fits may have been due to the instrumental temperature resolution of 0.01°C and the loss of high-frequency thermal changes resulting from the high thermal capacity of the system. These problems are most evident in the region where two peaks overlap and there are sudden changes in slope (see Fig. 4b). However, this fitting protocol was more reliable throughout the varying data acquisition conditions and calibration shifts than any other modeling techniques tested.

Discussion

The addition of agarose and galactose to concentrated samples of DPPC vesicles clearly broadens the chain melting transition of the lipid bilayers (see Fig. 5); separation into two different melting populations is evident at the 5.0% and 7.5% levels. Although Crowe and Crowe [16] reported the appearance of two endothermic components with the addition of glucose to unilamellar vesicles, they did not detect this effect in multilamellar vesicles. In our work, both of the endothermic components observed after addition of galactose to MLV melted at temperatures higher than T_m of hydrated DPPC. Agarose also broadens the phase transition into two components; however, only one of these components melts at an elevated temperature.

The low temperature peak found at high agarose concentrations coincides with the endotherm generated in the absence of the hydrogel. This suggests that the first peak is generated by lipids that are unaffected by the addition of agarose, possibly in the inner lamellae and inside the vesicles; the second peak may represent the melting of lipids whose environment is affected by agarose. In the concentration range of agarose studied, Table II and Fig. 6 indicate that the temperature shift of the higher endotherm component is constant while its relative area parallels the amount of agarose added. The simplest interpretation attributes agarose effects to some interaction with the most external monolayer, whether direct, via external water-structuring, or via external water-exclusion. Because of its high molecular weight, the osmotic effect of agarose should be much lower than that of an equivalent mass of galactose, unless the sulfate groups on the agarose contribute to an increase in the osmotic pressure. A dehydration effect would raise the melting temperature proportionally with increasing carbohydrate concentration. Since the high-temperature component did not further shift with higher agarose levels, the shifted population is most likely stabilized by some interaction with external agarose, as shown schematically in Fig. 8a. Such interaction can only affect lipids in the outer leaflet, which are a small fraction of the total lipids in MLV. Since the area in the second endotherm approaches 75% at 7.5 g/100 ml, either the enthalpy of the second population is significantly increased, or the

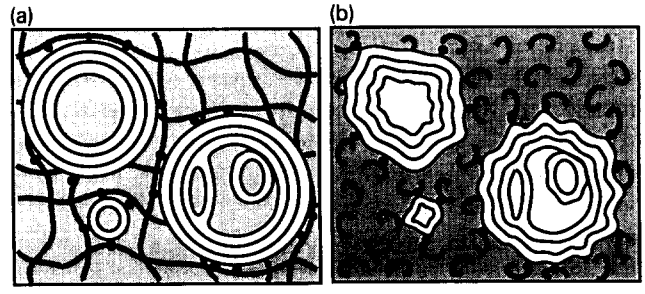

Fig. 8. Schematic representation of the effects of equal masses of polymer vs. monomer carbohydrates. In a, the high-molecular weight polymer carbohydrate (agarose) does not affect the osmotic pressure of the surrounding medium as much as in b, where the low-molecular weight monomer carbohydrate (galactose) induces an osmotic stress that partially dehydrates the interior lipids. The degree of interaction between carbohydrates and lipids in the external leaflets is assumed to be comparable in the two cases.

agarose is affecting a larger fraction of lipids than contained in outer leaflet.

Because galactose is the cleavage product of agarose, it should provide a similar chemical environment with three key differences: (1) it lacks the steric constraints of the agarose polymer, (2) it has two free hydroxyl groups that, in the polymer, are involved in covalent bonds, and (3) it is osmotically more active than the same mass of agarose, which might affect the hydration of internal lipids, as well as the compression on the entire liposome. As seen in the lower part of Table II, addition of galactose also broadened and split the total endotherm. However, the broadening is greater than with the same weight fraction of agarose in this set of results, and the low-temperature component melts at a higher temperature. This increase in the transition temperature is in agreement with earlier reports using MLV [10,12]. The peak of the high temperature component is significantly shifted from the control peak, by almost 0.6 C° in this set of scans. It is possible that stabilization of the outer monolayers in the presence of sugars is responsible for the less-shifted peak, while dehydration effects from the increased osmotic pressure cause a larger shift of the melting temperature of the internal lipids. Fig. 8b schematically depicts the effect of a high sugar concentration in the extraliposomal medium.

It has been suggested that the interaction between lipids and carbohydrates depends on the proportion of sugar to lipid, either in the form of mass ratio [20] or molar ratio [10,14]. This seems more relevant for cryopreservation, in which the sugar concentration ultimately becomes extremely high as it is excluded from ice crystals. A suggestion by Crowe that the effect of the sugar is correlated with its effect on the surface tension of the solution [16] is worth further investigation. It is conceivable that equivalent weight fractions of agarose and galactose may have nearly equivalent surface activities.

Table III reveals that the effect of adding agarose before vesicle formation is the same as when added after. If, as Gruner et al. suggested, it is unlikely that sugar in the hydrating buffer is incorporated into the interior of MLV [15], then in neither case is there solute inside, and the results should be the same. Other possible explanations for this negative result may be that thermal cycling allows vesicles to incorporate some of the polymer, or the water activity inside the liposomes is relatively unaffected by the presence of agarose inside the vesicles. The amount of sugar inside the vesicle depends partly on the ability of the mechanical agitation and thermal fluctuations to break apart and fuse liposomes. Further study of the intravesicular content of MLVs and changes with thermal cycling through the lipid phase transition might elucidate this issue.

In the galactose studies, the only difference in effect of adding sugar before vortexing is the decreased fraction of lipid in the high-temperature component. This is consistent with the idea that increasing the number of sugar molecules inside the vesicles would increase the number of lipids interacting with sugars directly (increasing the size of the lower temperature component) and reduce the osmotic gradient, leading to less dehydration. It is important to differentiate between the number of lipids that are partially dehydrated and the degree of dehydration of those lipids. If addition of galactose before vesicle formation increased the degree of dehydration, then the temperature of the second peak would be lowered, not the number of lipids contributing to its size. Therefore, it is likely that galactose induces a partial dehydration that affects all the lipids in the system; it also divides the endotherm into two populations through a direct interaction, similar to that caused by agarose.

Although we can not comment on enthalpy effects, the melting temperature is lowered by increasing the amount of Laurdan to 2.6 mol% and 5.4 mol%, as is expected of a small hydrophobic molecule buried in the bilayer. The only other effect is a slight change in the endotherm shape. Unlike in the carbohydrate studies, where a single suspension of vesicles can be combined with different sugar concentrations, each change in dye concentration requires preparation of a separate aqueous lipid/dye suspension. Reproducibility problems associated with the preparation process could contribute to the subtle difference in endotherm shape. The lack of significant effect of Laurdan up to 5 mol% was corroborated by another laboratory (Gratton, E., personal communication).

In conclusion, we have shown that the presence of agarose or galactose around vortexed large multilamellar vesicles of DPPC divides the chain melting endotherm into two components, ostensibly from two separately melting lipid species. We have also confirmed reports that addition of small carbohydrates to large multilamellar vesicles raises the chain melting transition temperature. Although we are unable to ascertain the mode of interaction, we suggest that separation occurs through a partial stabilization by interaction with lipid headgroups and that dehydration is responsible for the increase in temperature of the entire melting event. Finally, it appears that the fluorophore Laurdan is a non-perturbing probe of bilayer state, and at the levels used in our anesthetic sensor, any effects of Laurdan or agarose are insignificant.

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