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Solubilization of DMPC and DPPC vesicles by detergents below their critical micellization concentration: high-sensitivity differential scanning calorimetry, Fourier transform infrared spectroscopy and freeze-fracture electron microscopy reveal two interaction sites of detergents in vesicles

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The interaction of sodium deoxycholate, sodium cholate and octyl glucoside with sonicated vesicles of L_α-dimyristoylphosphatidylcholine (DMPC) and L_α-dipalmitoylphosphatidylcholine (DPPC) at concentrations below the critical micellization concentration (cmc) of the detergents was studied by high-sensitivity DSC (hs-DSC), Fourier transform infrared spectroscopy (FT-IR) and freeze-fracture electron microscopy. The two phospholipids exhibited a striking different thermotropic behaviour in the presence of these detergents. For DPPC vesicles, the detergents were found to interact exclusively in the aqueous interface region of the bilayer below the membrane saturation concentration R_{sat} while in DMPC vesicles two coexisting interaction sites below this concentration persist. These are detergents which interact at the aqueous interface region (site 1) and in the acyl chain region (site 2) of the DMPC vesicles. The partition coefficients K of the detergents between DPPC vesicles and the water phase were calculated from the hs-DSC results at two detergent/phospholipid molar ratios $R_{tot} \leqslant R_{sat}$ as 0.35, 0.049 and 0.040 mol^{-1} for sodium deoxycholate, sodium cholate and octyl glucoside, respectively. In contrast, for DMPC the K values for $R_{tot} \leqslant R_{sat}$ were found to be dependent on R_{tot} due to the occupation of site 2 by the detergents above a certain R_{tot}. The model is discussed on the basis of the detergents free energies of transfer from the water phase to site 1 and site 2 of the vesicles, respectively. The solubilization behaviour of DPPC vesicles, dependent on whether the total detergent concentration is above or below the cmc at R_{sat}, differed significantly as revealed by hs-DSC. This suggests that in the latter case an additional hydrophobic effect could facilitate the formation of disc shaped mixed micelles. Moreover, this different behaviour was employed to measure the cmc values of the detergents studied in the presence of the vesicles by hs-DSC.

Introduction

Solubilization of proteins and lipids by detergents followed by detergent dialysis is a widely used method for the reconstitution of integral membrane proteins into unilamellar vesicles (for review, see Ref. 1). The process of the formation of vesicles during dialysis and their physical and biochemical properties has been studied in recent years by various methods and theoretical considerations which improved considerably the understanding of this complex matter [2–12].

One shortcoming of the detergent dialysis method is that a certain amount of detergent remains in the vesicles even after extensive dialysis which can be reliable quantified by radioactive labelling only. This residual detergent can greatly obscure the interpretation of the measurements by various physical and biochemical methods on reconstituted vesicles making it difficult to distinguish between protein and detergent effects on the phospholipids. For example, 2% (mol) of the widely used detergent sodium deoxycholate affects already re-

Abbreviations: DMPC, L_α-dimyristoylphosphatidylcholine; DPPC, L_α-dipalmitoylphosphatidylcholine; T_m, gel to liquid-crystalline phase transition temperature; hs-DSC, high-sensitivity differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; cmc, critical micellization concentration.

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markably the phase transition temperature of phosphatidylcholine vesicles (cf. results in this work). In order to cope with that problem a better knowledge about the mechanism and the sites of interaction of the detergents with phospholipid vesicles is crucial, in particular at very low detergent concentrations. One important question is if at very low detergent concentrations the latter mix with the phospholipids in the bilayer of the vesicles or rather adsorb at the vesicles surface. This has important consequences for the physical chemistry of the vesicles surface.

Another intriguing question is whether the partial solubilization of a vesicle (i.e., vesicle rupture) can occur at detergent concentrations below its cmc where there is no coexistence between detergent micelles and vesicles and if such processes exist, whether they differ from those occurring above the cmc.

Most experiments conducted hitherto on phospholipid vesicles at very low detergent concentrations employed dynamic light scattering and optical density measurements [7–10]. Other solubilization studies were performed by measuring the release of water-soluble molecules from the interior of vesicles due to the interaction with detergents, mainly by fluorescence [11,12]. However, all these methods cannot give direct information on the intrinsic detergent–phospholipid interaction at concentrations below the rupture of the vesicles due to solubilization and are in addition prone to errors by vesicles aggregation. Some studies were performed with nuclear magnetic resonance (NMR) methods [8,10,13–15] but they require high sample concentrations, mostly above the cmc of the detergents, except for proton NMR. However, the latter method suffers from incomplete motional averaging of the vesicle constituents on the NMR timescale. The concomitant broadening of the signals reduces its potentially high sensitivity for such studies.

High-sensitivity differential scanning calorimetry (hs-DSC) is well established as the most powerful method to study the thermotropic behaviour of saturated phospholipids (for review, see Ref. 16). Its sensitivity allows reliable measurements of phospholipid vesicles at concentrations below 0.7 mM. The phase transition behaviour of vesicles depends on several parameters such as intermolecular forces, bending energy and molecular packing constraints but is largely independent on vesicle aggregation and size changes above a critical diameter. These features recommend hs-DSC for the study of interactions between vesicles and detergents at very low concentrations. It was already successfully employed to the study of disc shaped micelles of bile salts and phosphatidylcholines [4].

Fourier transform infrared spectroscopy (FT-IR), which is well established in the study of lipids [17,18], provides in combination with the attenuated total reflection (ATR) technique for liquids another powerful tool of high sensitivity for the study of such systems. It has been applied in order to study the interaction of phosphatidylcholines with Triton X-100 [19] but at concentrations which were well above the cmc of this detergent.

In the present work the interaction of the sodium salts of cholic and deoxycholic acid as well as of octyl glucoside with vesicles of DMPC and DPPC was studied at various detergent to lipid molar ratios below the cmc of the detergents by hs-DSC, FT-IR and freeze-fracture electron microscopy. The detergents were selected for their widely use in protein reconstitution. As the bile salts possess steroid bodies similar to cholesterol, their effects on the thermotropic behaviour of the vesicles was compared with that of the latter in order to obtain additional information about their interaction sites in the bilayer. The cmc values of the detergents in the presence of the vesicles (which are expected to be different from that in pure water) were measured by hs-DSC. Moreover, some hs-DSC measurements on these systems were performed at higher concentrations of the mixtures (well above the cmc of the detergents) in order to compare the solubilization behaviour with that at low concentrations. The results strongly suggest the existence of two interaction sites of detergents in phosphatidylcholine vesicles and indicate remarkable differences in the solubilization behaviour depending on whether it takes place above or below the cmc of the corresponding detergent.

Materials and Methods

Vesicle preparation

The L_α-phosphatidylcholines DMPC and DPPC were purchased from Avanti Polar Lipids. The lipids were checked for purity by thin-layer chromatography before use. The lipids were dispersed in 50 mM Hepes (pH 7.0), 2 mM EDTA (buffer I), at 10 °C above its gel to liquid-crystalline phase transition temperature (T_m) and incubated at this temperature for 3 h. Then, the lipid dispersions were sonicated at this temperature using a Branson Tip sonifier (15 min at 30 W, pulsed mode with 50% duty cycle). In order to remove lipid aggregates and titanium dust, the vesicles were centrifuged at $10000 \times g$ for 10 min. Finally the suspensions were five times frozen and slowly thawed in order to fuse very small vesicles. If not stated otherwise, the typical phospholipid concentration in the samples was 1.5 mM. The mean hydrodynamic diameter of the vesicles was measured by dynamic light scattering between 65 and 70 nm.

Vesicles of DPPC with cholesterol were prepared by dissolving the lipid mixture with cholesterol in chloroform. The solvent was evaporated under a stream of nitrogen followed by overnight vacuum desiccation. The lipid film was taken up in buffer I and further processed as described above.

The detergents used were obtained from Serva (deoxycholic acid sodium salt, octyl β-D-glucopyranoside) and Sigma (cholic acid sodium salt). The bile salts were purified by dissolving in ethanol, filtering and recrystallization. They were dissolved in buffer and added to the vesicles at a temperature of 10 °C above the T_m of the lipids and finally incubated at this temperature for at least 16 h. This incubation was allowed in order to ensure a homogeneous distribution of the detergents over the sample. However, it should be emphasized that our hs-DSC measurements indicated that a stable distribution of all detergents studied was already reached after 20 min incubation time, i.e. the hs-DSC scans were independent on incubation time after this period for at least 35 h. Even the use of multilamellar (nonsonicated) vesicles in control experiments exhibited completely equilibrated hs-DSC endotherms 1 h after the detergent addition. This finding is in disagreement with observations made by Schurtenberger et al. [7], where equilibration times of the order of 48 h were needed for dynamic light scattering measurements. The reason for that are probably the basic differences between the methods applied (hs-DSC and dynamic light scattering).

hs-DSC measurements

High-sensitivity DSC measurements were performed with an MC-2 (Microcal, Amherst, MA) microcalorimeter interfaced to an IBM AT microcomputer. The data were stored and analyzed by this computer using the DA-2 software provided by Microcal.

The samples were transferred to a cooling bath and equilibrated at the starting temperature of the hs-DSC scan for 5 min and then filled into the calorimeter. The heating scan was started after additional 15 min equilibration with a scan rate of 50 C°/h and a 10 s time increment (filter constant) between the data acquisitions. Control measurements were also performed at a 20 C°/h scan rate and gave identical results. Selected samples of DMPC and DPPC with sodium deoxycholate were studied in the descending temperature mode using the MC-2 cooling scan unit at a scan rate of −10 C°/h. No significant differences as compared to the endotherms obtained by heating scans were observed.

In order to avoid any effects of phospholipid hydrolytic breakdown products, the maximum storage time of the vesicles-detergent mixtures was 35 h.

The transition enthalpies ΔH were determined after subtraction of the buffer baseline. The integration limits were defined by connecting the regions of flat baseline with a straight line. This and the integration were doen via a subroutine of the DA-2 program.

The transition temperatures (T_m) of the pure vesicles were obtained from the specific heat maximum of the endotherms. For asymmetric lineshapes of the composite vesicles the transition temperature (T_m) was defined as that temperature at which the area of the endotherm was divided into two equal halves.

Control experiments were also performed with large multilamellar vesicles (prepared by gentle shaking of the swollen phospholipids in buffer). All results reported below for sonicated vesicles were found to be qualitatively similar in multilamellar vesicles, in particular the changes of T_m and ΔH upon detergent addition.

FT-IR measurements

For FT-IR measurements a Nicolet 60 SXB spectrometer equipped with a liquid ATR accessory (thermostated circle cell with ZnSe crystal from Spectratech) and an MCT detector was used. The ATR unit allowed a good signal to noise ratio at low phospholipid concentrations (1.5 mM as in the other experiments). In order to prevent interference of the water bands and the methylene stretching modes of the phospholipids, the samples were dissolved in buffered D_2O The temperature was measured in the circle cell using a Pt 100 thermocouple which controlled an external water bath and was constant within ±0.2°C. The measurements were performed from lower to higher temperatures for each sample, at each temperature 600 scans were accumulated at a resolution of 2 cm^{-1} between 4000 and 400 cm^{-1}. The buffer spectra were recorded separately at the same temperatures and interactively subtracted from the sample spectra. The frequency of the methylene stretching vibrations was determined as the maximum of the corresponding bands.

Electron microscopy

Freeze-fracture electron micrographs of the samples used for hs-DSC measurements were taken for pure DPPC and DMPC vesicles as well as for those treated with sodium deoxycholate at different concentrations. The samples were jet frozen from ambient temperature using a home-built device in liquid nitrogen, processed in a Balzers freeze-fracture device and observed using a Phillips EM 400T electron microscope.

Results

1. hs-DSC measurements

As a matter of convention, the following quantities were used in the text in order to express the amounts of phospholipids and detergents in the samples. These are

$$R_{tot} = [D_{tot}]/[L],\ x_{tot} = [D_{tot}]/([L]+[D_{tot}])\ \text{and}$$

$$x_{eff} = [D_{tot}]/([L]+[D_{tot}]+K^{-1}) \quad (1)$$

where [L] is the phospholipid concentration, $[D_{tot}]$ is the total detergent concentration and K is the partition coefficient as defined in Eqn. 2.

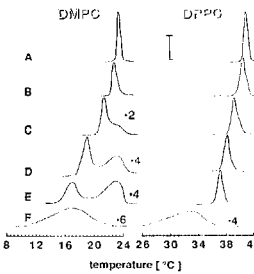

Fig. 1. hs-DSC excess heat capacity profiles of unilamellar vesicles of DMPC (left column) and DPPC (right column) with sodium deoxycholate at different molar ratios, $R_{tot} = [D_{tot}]/[L]$, or different effective molar fractions of sodium deoxycholate, x_{eff}. $R_{tot} = \cdot$, ($x_{eff} = \cdot$) A: 0, (0); B: 0.05, (0.017); C: 0.20, (0.065); D: 0.33, (0.104); E: 1, (0.258); F: 2, (0.410). The total phospholipid concentration was [L] = 1.5 mM. Some hs-DSC profiles were increased in intensity by the factors at the right of the endotherms. The bar represents 1 kcal/K per mol.

Fig. 1 shows the hs-DSC endotherms of small unilamellar vesicles of DMPC and DPPC with different proportions (R_{tot}) of sodium deoxycholate.

For DMPC, this detergent causes first at low R_{tot} a slight broadening of the endotherms together with a low-temperature shift of the chain melting transition temperature T_m (Fig. 1A, B). The latter decreased linearly with increasing R_{tot}. Above $R_{tot} \approx 0.15$, a second broader signal appeared from a flat high-temperature shoulder of the endotherm (Fig. 1C), with a maximum located at 0.5°C below the T_m for pure DMPC vesicles (24.1°C). Increasing R_{tot} continued to shift the narrow peak towards lower temperatures but simultaneously its intensity is reduced at $R_{tot} > 0.5$. The maximum of the broad signal remains constant at 23.6°C up to $R_{tot} = 0.95$ (Fig. 1D, E). Above this concentration the narrow peak gradually vanishes and the broad signal at 23.5°C is progressively broadened and shifted towards lower temperatures with increasing R_{tot} (Fig. 1F).

The behaviour of DPPC vesicles is strikingly different as there is no splitting of the excess heat capacity profile upon sodium deoxycholate addition but only a slight broadening and a continuous shift of the T_m towards lower temperatures linearly with R_{tot} (Fig. 1B–E and Fig. 4B). However, at $R_{tot} = 1.1$ (corresponding to a T_m of the endotherm of 37.0°C) there is an abrupt change of the shape of the endotherm into a broad and asymmetric profile which continues to shift towards lower temperatures for even higher R_{tot} (Fig. 1E, F).

The effect of sodium cholate was qualitatively similar to that of sodium deoxycholate on DMPC and DPPC, but the concentrations required to cause a shift of T_m were higher and the magnitude of the low-temperature shift of the endotherms upon sodium cholate addition was less than for sodium deoxycholate.

Octyl glucoside, a nonionic detergent with a bulky hydrophilic sugar headgroup and a comparatively small alkyl chain, exerts a similar effect on DPPC as the bile salts but at considerably higher R_{tot}. Its effect on DMPC differs remarkably from that reported above for the bile salts as it gave no rise for a broad second endotherm at 23.6°C. Instead, the endotherm broadens slightly upon octyl glucoside addition (Fig. 2B, C), shifts towards lower temperatures and eventually splits into a narrow and a broad signal at $R_{tot} > 4$ (Fig. 2D). This broad part of the endotherm, however, is centered well below ($\Delta T_m = -8.2$°C) the initial T_m of 24.1°C for pure DMPC. The next steps are similar to that reported above for bile salts, the narrow signal proceeds to shift to lower temperature, reduces in intensity (Fig. 2E) and eventually vanishes with increasing R_{tot}. Then the broad part of the endotherm shifts from 15.9°C towards lower temperatures at even higher R_{tot} ($R_{tot} > 8.3$). The transition enthalpy ΔH of the whole transition endotherm slightly decreases from 6 kcal/mol down to 5.5 kcal/mol for the DMPC/sodium deoxycholate vesicles with increasing R_{tot} up to $R_{tot} = 0.5$ (Fig. 3). A similar slight decrease of ΔH by 10–15% was observed for DMPC with sodium cholate and octyl glucoside, respectively. In contrast, for DPPC vesicles with sodium deoxycholate, ΔH is constant at the value for pure DPPC vesicles (8 kcal/mol) up to $R_{tot} = 1.1$ (Fig. 3).

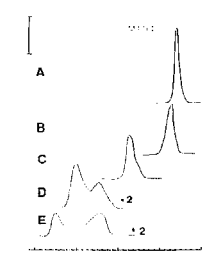

Fig. 2. hs-DSC excess heat capacity profiles of DMPC unilamellar vesicles with octyl glucoside at different molar ratios, $R_{tot} = [D_{tot}]/[L]$, or different effective molar fractions of octylglucoside, x_{eff}. $R_{tot} = \cdot$, ($x_{eff} = \cdot$) A: 0, (0); B: 0.05, (0.0028); C: 2, (0.122); D: 5, (0.277); E: 8, (0.385). The total phospholipid concentration was [L] = 1.5 mM. The endotherms D, E were increased by a factor of 2 in intensity. The bar represents 1 kcal/K per mol.

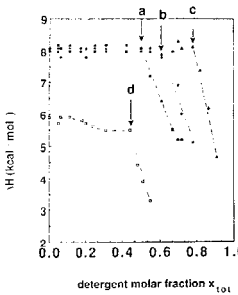

Fig. 3. The transition enthalpy ΔH versus the total detergent molar fraction x_{tot} ($x_{tot} = [D_{tot}]/([D_{tot}]+[L])$) for unilamellar vesicles of DPPC (upper three traces) and of DMPC (lower trace): (■) sodium deoxycholate, (◆) sodium cholate, (▲) octyl glucoside. The total phospholipid concentration was [L] = 1.5 mM. The arrows represent the membrane saturation concentrations (R_{sat}): a, b, c for sodium deoxycholate, sodium cholate and octyl glucoside, respectively, in DPPC vesicles and d for sodium deoxycholate in DMPC vesicles.

These detergent concentrations R_{tot} coincide with those at which the drastic broadening of the endotherm for DPPC (Fig. 1D) and the onset of the decrease of the narrow low-temperature part of the endotherm for DMPC (Figs. 1E and 2E), respectively, can be observed. Above these concentrations, there is a drastic change of ΔH for DMPC and DPPC vesicles over a relatively narrow concentration increment of R_{tot} down to a new value which is 35–45% less than the initial ΔH (Fig. 3). From this minimum the ΔH increases at even higher R_{tot}. These drastic change of the transition enthalpy ΔH of DMPC and DPPC is similar for all three detergents studied in this work. The critical detergent concentrations above which the change of ΔH occurs are different and characteristic for each detergent.

In order to obtain additional information about the effect of the phospholipid chain length on the features of the hs-DSC endotherms, some measurements of DSPC vesicles ([L] = 1.5 mM) with sodium deoxycholate were performed. The results are qualitatively similar to those reported for DPPC, but ΔH is constant at its initial value at even higher detergent concentrations than for DPPC (up to $R_{tot} \approx 1.6$).

Dependence on the total phospholipid concentration, partition coefficient

The detergent effects on T_m and ΔH of DMPC and DPPC showed a characteristic dependence on the total phospholipid concentration, owing to the partition of detergent molecules between bilayer and aqueous bulk phase. According to Schurtenberger et al. [7], a partition coefficient K for this process has been defined as

$$K = [D_B]/([L][D_m]) = R_{eff}/[D_m] \quad (2)$$

and

$$[D_{tot}] = [D_B] + [D_m] \quad (3)$$

where [L] and $[D_{tot}]$ are the total lipid and detergent concentrations, respectively, $[D_m]$ is the monomeric detergent concentration in the aqueous bulk phase and $[D_B]$ is the concentration of detergent associated in the bilayer of the vesicles. R_{eff} is the effective molar ratio of detergent to lipids in the vesicles. Thus, the concentration dependence of R_{eff} at which a certain T_m of the endotherm is observed can be used to evaluate the partition coefficient K at this T_m (R_{tot}). Assuming that the dependence of T_m on [L] and $[D_{tot}]$ is a monotonic function of the effective detergent to lipid molar ratio, R_{eff}, in the bilayer of the vesicles, the plot of the different sets of [L] and $[D_{tot}]$ which yield the same T_m gives a straight line for each detergent/vesicle mixture (Fig. 4A). This line can be described by the following expression

$$[D_{tot}] = [D_m] + R_{eff}[L] \quad (4)$$

Division of R_{eff} by $[D_m]$, obtained from plots like that in Fig. 4A yields the partition coefficient K for each detergent as defined in Eqn. 2. This partition coefficient should be constant for all $R_{tot} < R_{sat}$ (R_{sat} is the detergent to lipid molar ratio above which the vesicle ruptures), provided that there is only one interaction site of the detergents in the vesicles. Thus, the calculation of K for different T_m values allows us to determine whether the partition of detergents between vesicles and bulk water can be described by a single partition coefficient. The values of $R_{eff} = [D_B]/[L]$, $[D_m]$

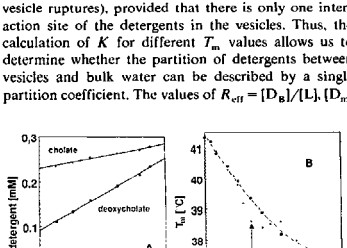

Fig. 4. (A): Plots of different sets of [L] and $[D_{tot}]$ which give a phase transition temperature $T_m = 40.3°C$ of the hs-DSC endotherm for DPPC vesicles with sodium deoxycholate (■) and sodium cholate (◆). The values R_{eff} and $[D_m]$ as presented in Table I were obtained from such plots as the slope of the fitted line (R_{eff}) and its intercept with the D_{tot} axis ($[D_m]$). (B): The phase transition temperature T_m of DPPC vesicles with sodium deoxycholate versus R_{tot} at two different DPPC concentrations [L] = 1.5 mM (■) and at [L] = 10.8 mM (+).

TABLE I

Partition coefficients K, monomeric detergent concentrations $[D_m]$ and an effective ratio of detergent to phospholipid in the vesicles, R_{eff}, calculated according to Eqn. 4 for sodium deoxycholate, sodium cholate and octyl glucoside in DMPC and DPPC vesicles at two T_m values

The unitary free energies of transfer between the water phase and the vesicles interface $(\mu^0_w - \mu^0_{if})/RT$ or acyl chain region $(\mu^0_w - \mu^0_{hc})/RT$ were calculated according to Equations 6 and 9, respectively (cf. Discussion).

Lipid	Detergent	T_m (°C)	$[D_m]$ (mM)	R_{eff}	K (l/mM)	$(\mu^0_w - \mu^0_{if})/RT$	$(\mu^0_w - \mu^0_{hc})/RT$
DMPC	sodium deoxycholate	23.1	0.095	0.031	0.32	−0.71	
		16.5	0.64	0.34	0.53	"	−1.15
DMPC	sodium cholate	23.1	0.23	0.011	0.048	2.63	"
		20.4	1.37	0.081	0.058	"	−4.19
DMPC	octyl glucoside	23.1	0.14	0.006	0.041	−2.75	"
		14.5	6.9	0.420	0.057	"	−3.80
DPPC	sodium deoxycholate	40.3	0.095	0.032	0.34	−0.68	"
		37.0	0.79	0.278	0.35	"	"
DPPC	sodium cholate	40.3	0.23	0.011	0.048	−2.63	"
		38.8	1.45	0.071	0.049	"	"
DPPC	octyl glucoside	40.3	0.15	0.006	0.040	−2.81	"
		26.9	9.9	0.35	0.040	"	"

and K calculated from the experimental data for the three detergents studied in DMPC and DPPC vesicles at two different T_m values (corresponding to two R_{tot} values) are given in Table I.

It is obvious from Table I that K is similar for DMPC and DPPC at low R_{tot} (compare the K values at the $T_m = 23.1$°C (DMPC) and 40.3°C (DPPC)) for the same detergent. However, at $R_{tot} \approx R_{sat}$ corresponding to T_m values at which the above-mentioned drastic broadening of the endotherms and the sharp decrease of ΔH can be observed, the K value is considerably increased for DMPC while it remains constant for DPPC. This finding is independent on the detergents used.

Measurement of the critical micellization concentrations (cmc)

The relatively linear shift of the T_m with increasing R_{tot} of DMPC and DPPC vesicles (cf. Fig. 4B) was used to measure the cmc of the detergents studied in the presence of the vesicles. The knowledge of these values is essential in order to decide if the measurements reported above were performed strictly below the cmc. The cmc can be measured by choosing a phospholipid concentration [L], which is sufficiently high to ensure that the concentration of the detergent to be added to the vesicles will exceed its cmc below the membrane saturation concentration R_{sat}. The hs-DSC endotherms measured under this conditions at various detergent to lipid molar ratios $R_{tot} = [D_{tot}]/[L]$ where [L] was kept at constant high concentration (e.g., [L] = 10.8 mM for DPPC vesicles with sodium deoxycholate) do not exhibit a simple linear decrease of T_m with R_{tot} but a plateau region or even a slight local increase of T_m as shown in Fig. 4B. This plateau region of T_m is caused by the onset of the formation of detergent micelles in the aqueous bulk solution. This changes the partition coefficient K of the detergents between vesicles and the aqueous bulk phase in favour of the latter and causes thus a depletion of detergent associated with the vesicles until the micelles have reached an energetically favorable aggregation number. After the new equilibrium partition coefficient K is established, further detergent addition continues to decrease the T_m of the vesicles. The cmc values of the detergents used in this work were extrapolated from the concentration $[D_{tot}]$ at the beginning of the T_m plateau region (indicated by an arrow in

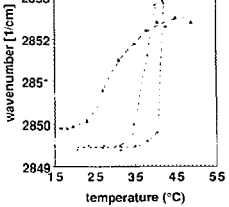

Fig. 5. Symmetric methylene stretching vibration wavenumbers of DPPC fatty acid chains versus temperature of DPPC vesicles without (+) and with sodium deoxycholate. $R_{tot} = (x_{eff} =)$: 0.33 (0.104) (■) and 2.0 (0.410) (▲).

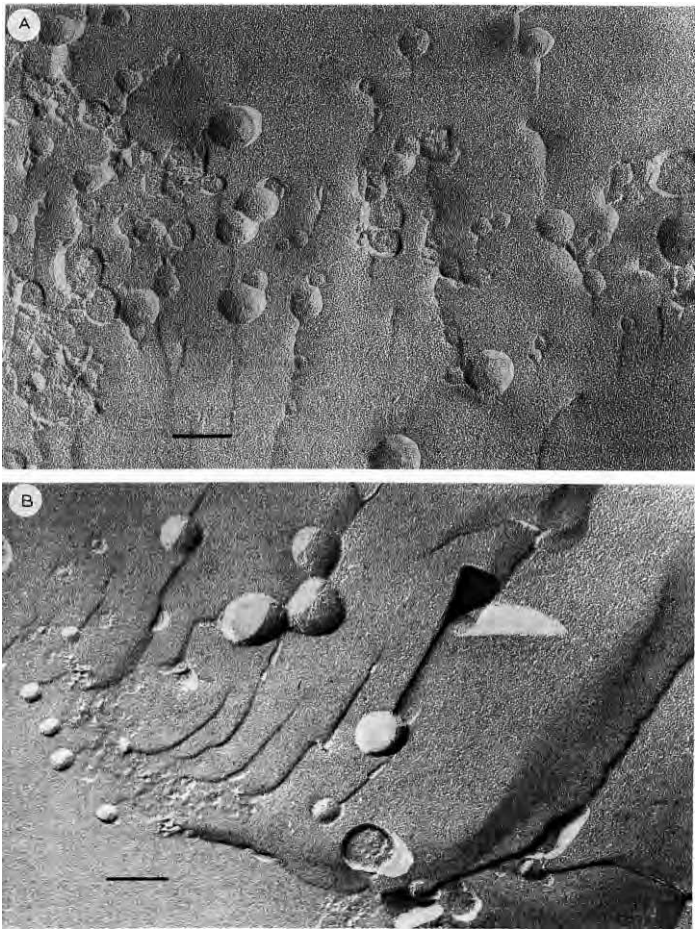

Fig. 6. Freeze-fracture electron micrographs of DPPC vesicles without (A) and with (B) sodium deoxycholate ($R_{tot} \approx 0.80$). The DPPC concentration was [L] = 1.5 mM. The bar represents 0.1 μm.

Fig. 4B) as 2.2 mM (sodium deoxycholate), 3.2 mM (sodium cholate) and 18 mM (octyl glucoside).

2. Fourier transform infrared measurements

FT-IR measurements were performed in order to obtain information about the interaction sites of sodium deoxycholate in DPPC vesicles below and above R_{sat} at total detergent concentrations $[D_{tot}] <$ cmc of sodium deoxycholate. As we were mostly interested to answer the question if there is an interaction of sodium deoxycholate with the hydrophobic interior of the DPPC vesicles below R_{sat}, the methylene stretching vibrations of the DPPC fatty acyl chains were measured as a function of temperature.

Fig. 5 shows the temperature dependence of the symmetric methylene stretching vibrations for DPPC vesicles alone and with sodium deoxycholate below and above R_{sat}. The frequency of this band is related to the average number of *gcuche* conformers in the vesicles. This frequency changes for pure DPPC vesicles from 2849.6 cm^{-1} (*all-trans*-conformation) to 2853.0 cm^{-1} above 45°C due to the introduction of *gauche* conformers. This difference of 3.4 cm^{-1} is caused by the melting of the acyl chains of DPPC at the gel to liquid-crystalline phase transition.

The addition of sodium deoxycholate at a molar ratio $R_{tot} = 0.33$ (which is below R_{sat}) lowered T_m by $\approx 3°C$ but did not significantly broaden the transition, in agreement with the hs-DSC results. The total frequency difference of 3.4 cm^{-1} between gel and liquid-crystalline state was not changed.

In contrast, at $R_{tot} = 2$ (which is above R_{sat} but still below the cmc) one can observe a drastic broadening of the transition and a reduction of the frequency difference between both states down to 2.7 cm^{-1}. It is obvious that the average number of *gauche* conformers was increased in the gel state and decreased in the liquid-crystalline state at this detergent concentration. The drastic broadening of the phase transition is again in excellent agreement with the hs-DSC results while the change of the number of *gauche* conformers in both states can be related to the reported drastic change of the transition enthalpy ΔH above R_{sat}.

3. Freeze-fracture electron microscopy

Freeze-fracture electron microscopy measurements were performed on pure DMPC and DPPC vesicles and at various molar ratios of sodium deoxycholate above and below R_{sat}. In Fig. 6 freeze-fracture replicas of DPPC vesicles without (Fig. 6A) detergent and with sodium deoxycholate where R_{tot} is just below R_{sat} (Fig. 6B) are shown. It should be emphasized that $[D_{tot}]$ is nevertheless well below the cmc of sodium deoxycholate in Fig. 6B. A comparison between Figs. 6A and B shows that no significant morphological changes of the vesicles with sodium deoxycholate as compared to the pure DPPC vesicles can be observed at $R_{tot} < R_{sat}$. The results for DMPC vesicles are similar, there is in particular no indication for the coexistence of two vesicles populations at $R_{tot} < R_{sat}$, as the splitting of the DSC endotherms (Fig. 1) might suggest.

In contrast, at concentrations of sodium deoxycholate $R_{tot} > R_{sat}$ ($R_{tot} = 2$ for both DMPC and DPPC vesicles) only smooth replicas without any vesicles or mixed micelles were found (not shown). Possibly the mixed micelles are too small at this R_{tot} and can not be resolved.

Discussion

The interaction of detergents with phospholipid vesicles is mostly discussed in the frame of the three-stage model of solubilization as proposed by Lichtenberg et al. [6]. This model postulates the incorporation of detergent monomers into the phospholipid bilayer as the first stage of solubilization. The second stage is then the formation of phospholipid-detergent mixed micelles which occurs above a certain critical detergent to lipid ratio in the bilayer, called the membrane saturation concentration R_{sat}. The coexistence of mixed micelles with vesicles above R_{sat} is determined by the balance of the vesicles bending energy with the edge tension of the mixed micelles [5]. In the third stage of solubilization, upon exceeding a detergent to lipid molar ratio in the bilayer called the membrane solubilization concentration, R_{sol}, the coexistence of lamellar and mixed micellar structures ceases to exist and all phospholipids are present in mixed micelles.

The most striking difference between DPPC and DMPC vesicles is the splitting of the endotherms of the latter upon addition of detergents (Fig. 1,2). This splitting was observed for all detergents studied at different R_{tot}.

The considerable increase of the partition coefficient K with decreasing T_m (i.e., increasing R_{tot} at $[L] =$ const.) which was found for DMPC but not for DPPC (Table I) represents a further significant difference. It suggests that both phospholipids accommodate approximately the same amount of detergent molecules at low R_{tot} while at higher R_{tot} DMPC vesicles can interact with more detergent than DPPC.

The splitting of the hs-DSC endotherms for DMPC can be explained by an additional interaction site of the detergents in DMPC vesicles which is occupied at higher R_{tot} which is still below R_{sat}. The origin for this behaviour is very likely the weaker chain–chain interaction of the latter, because the two carbons shorter fatty acyl chains of DMPC. The most likely interaction sites are the interface region (site 1) and the hydrocarbon region (site 2) as schematically drawn in Fig. 7.

In terms of this model the narrow hs-DSC signal where T_m varies linearly with R_{tot} is caused by the

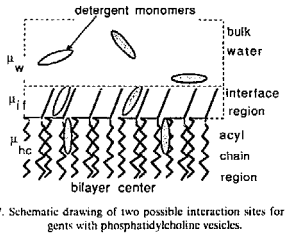

Fig. 7. Schematic drawing of two possible interaction sites for detergents with phosphatidylcholine vesicles.

interaction of detergents at the aqueous interface region of the vesicles (site 1). The broad part of the endotherm observed for DMPC at higher R_{tot} is caused by detergents incorporated into the fatty acyl chain region of the bilayer (site 2).

The partition of the detergents between site 1 and site 2 in the vesicles is determined by the chemical potential difference between the detergents in the water phase and the two interaction sites in the vesicles. As regards the interface-interacting detergents this energy difference can be estimated by assuming that the chemical potential of the detergent monomers in the water phase (μ_w) equals that of the detergent in the interface of the vesicle (μ_{if}) at equilibrium as follows [20].

$$\mu_w^0 - \mu_{if}^0 = RT \ln(x_{if}/x_w) + RT \ln(f_{if}/f_w) \quad (5)$$

where the term on the left is the free energy difference, x_{if}/x_w is the partition of detergent between water and vesicles in mol fraction units and f_w, f_{if} are the activity coefficients. The latter can be assumed to be unity and thus $\ln(f_w/f_{if}) = 0$. Hence, $\mu_w^0 - \mu_{if}^0$ can be obtained directly from the partition coefficient K, where $x_{if}/x_w = [D_B]/[D_w]$.

The calculated values of the unitary free energies of transfer are presented in Table I. These energies are significantly lower for sodium deoxycholate than for the other detergents because the former possess the lowest number of hydrophilic groups of the detergents studied. Moreover, the very similar values of these energies for both phospholipids indicates that there is no difference in the interaction at low R_{tot}. This is also indicated by the similar linear dependence of T_m on R_{tot} (cf. Fig. 4A for DPPC vesicles with sodium deoxycholate) for both phospholipids.

The increase of the partition coefficient K for DMPC at high values of $R_{tot} \approx R_{sat}$ can be expressed as

$$\Delta K = K_{sat} - K_1 \quad (6)$$

where K_1 is the value of K obtained at low R_{tot} and K_{sat} is the corresponding value at $R_{tot} \approx R_{sat}$. This difference ΔK is caused by the occupation of site 2 in the vesicles with detergent molecules. Thus

$$K_{sat} = K_1 + \Delta K = [D_{if}]/([L][D_m]) + [D_{hc}]/([L][D_m]) \quad (7)$$

where

$$[D_B] = [D_{if}] + [D_{hc}] \quad (8)$$

is the sum of the detergent concentrations at site 1 ($[D_{if}]$) and site 2 ($[D_{hc}]$) in the vesicles. Assuming now that $\mu_w = \mu_{if} = \mu_{hc}$ (cf. Fig. 8), $\mu_w^0 - \mu_{if}^0$ = constant (as observed for DPPC vesicles) and $[D_{if}] \gg [D_{hc}]$ so that the activity coefficients can be neglected (i.e., $\ln(f_{hc}/f_w) = 0$), we obtain an estimate for the free energy of transfer between the water phase and the hydrocarbon chain region (site 2) of the vesicles:

$$\mu_w^0 - \mu_{hc}^0 = RT \ln(x_{hc}/x_w) \quad (9)$$

These values were calculated for DMPC vesicles with

$$x_{hc}/x_w = [D_{hc}]/[D_m] = \Delta K [L] \quad (10)$$

and are represented in Table I. They are significantly higher than the corresponding free energies of transfer between site 1 and the water phase. This explains why an interaction with site 2 can be observed at higher R_{tot} only.

The magnitude of $\mu_w^0 - \mu_{hc}^0$ is mainly ruled by the intrinsic lateral pressure π_i of the vesicles bilayer which was shown to increase with increasing acyl chain length [21]. As a consequence, a considerably higher $\mu_w^0 - \mu_{hc}^0$ would be required for the interaction of detergent with site 2 in DPPC vesicles. Therefore for DPPC vesicles only one detergent interaction site (site 1) can be observed at $R_{tot} \ll R_{sat}$.

A salient feature in the thermotropic behaviour of all DPPC vesicles with detrgents is the unchanged transition enthalpy ΔH as compared to the value of ΔH of pure DPPC vesicles up to a certain $R_{tot} = R_{sat}$ above which an abrupt change of ΔH can be observed (Fig. 3). In contrast, for DMPC vesicles ΔH slightly decreases already below this detergent concentration.

We interpret the onset of the drastic ΔH change as the beginning of the coexistence of mixed micelles with vesicles above $R_{tot} = R_{sat1}$, i.e., the stage 2 of solubilization in the model of Lichtenberg et al. [6]. This assumption is supported by the results of other authors who conducted optical density measurements on these systems with similar results concerning R_{sat} [9,10]. Further evidence for this interpretation comes from the finding that this R_{sat} value corresponds to a T_m, which is constant irrespective the total phospholipid concentration [L] for each lipid-detergent mixture (e.g. for DPPC/sodium deoxycholate mixtures $T_m = 37.0\,^\circ C$ at

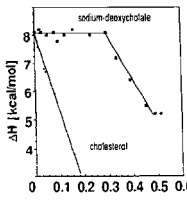

Fig. 8. The transition enthalpy ΔH versus the effective detergent molar fraction x_{eff} ($x_{eff} = [D_{tot}]/([D_{tot}] + [L] + K^{-1})$) for DPPC vesicles with sodium deoxycholate (■) and cholesterol (×), respectively. For cholesterol was $[D_{tot}]$ = total cholesterol concentration and $K^{-1} = 0$. The total phospholipid concentration was [L] = 1.5 mM.

R_{sat}, independent of [L]; (cf. Table I for the T_m values at R_{sat} of the other detergents).

The structure of the mixed micelles formed above R_{sat} has been described as lamellar sheets of phospholipids and detergent molecules which are surrounded on their perimeter by bile salts [2] or octyl glucoside [9]. Hence, the reported decrease in ΔH by 35–40% above R_{sat} (Fig. 3) corresponds to the ΔH difference of phspholipids arranged in vesicles and in mixed micelles, respectively.

The increase of ΔH after the transition from vesicles to mixed micelles at $R_{tot} > R_{sat}$ (cf. Results) was not considered in this work because this has been extensively discussed by Spink et al. [4].

The unchanged ΔH for DPPC and the slight but remarkable decrease of ΔH for DMPC ($R_{tot} < R_{sat}$) provides further support for the two site interaction model in Fig. 7. The interaction of bulky molecules like bile salts with the hydrocarbon region of the vesicles (site 2) is expected to decrease the transition enthalpy ΔH with the concentration of detergents at this site ($[D_{hc}]$). This is demonstrated for a molecule such as cholesterol which possesses a similar steroid body as sodium deoxycholate in Fig. 8. Its incorporation into DPPC vesicles causes a decrease of ΔH linearly with the cholesterol concentration as reported by Mabrey et al. [22].

Thus, the unchanged ΔH of DPPC vesicles with detergents at $R_{tot} < R_{sat}$ provides evidence that the only possible interaction site is the interface region (site 1). The interaction at this site changes mainly the phase transition temperature T_m by modification of the headgroup interactions but not the transition enthalpy ΔH, as the van der Waals interaction between the fatty acyl chains does not change significantly. Upon exceeding R_{sat}, the detergents enter into the hydrophobic region (site 2) of the vesicles and decrease ΔH by changing the basic interactions in this region as observed for cholesterol (Fig. 8).

In contrast, the slight decrease of ΔH for DMPC at $R_{tot} < R_{sat}$ is caused by that amount of detergents ($[D_{hc}]$) which interact with site 2 in the DMPC vesicles, in agreement with the splitting of the hs-DSC endotherm. A comparison of $\Delta(\Delta H) \approx 0.5$ kcal/mol for DMPC with sodium deoxycholate below R_{sat} (cf. Fig. 3) with the large effect of cholesterol on ΔH of DPPC (Fig. 8) shows that $[D_{hc}]$ is very low which justifies the assumption $[D_{if}] \gg [D_{hc}]$ made above.

The unchanged features of the hs-DSC endotherms obtained in the descending temperature mode as compared to those of the heating scans provide evidence that the reported behaviour of ΔH versus R_{tot} for $R_{tot} < d_{sat}$ cannot be caused by an exclusion of the detergents from the vesicles at the transition from the liquid-crystalline to the gel state. Furthermore, the FT-IR results give evidence for two different interaction sites of sodium deoxycholate in DPPC vesicles below and above R_{sat}. The shift of the T_m without broadening by sodium deoxycholate below R_{sat} and the unchanged number of *gauche* conformers between both states is compatible with an interaction of sodium deoxycholate at the interface region of the DPPC vesicles (site 1).

Above R_{sat}, the significant changes of the symmetric methylene stretching vibrations versus temperature are similar to those reported for cholesterol in DPPC multilayers [23]. This is in agreement with the hs-DSC measurements presented in Fig. 8 and allows the conclusion that a part of the DPPC fatty acyl chains are retained in the *all trans* conformation in the liquid-crystalline state due to the immediate neighborhood of sodium deoxycholate molecules. This could be DPPC molecules interacting with sodium deoxycholate at the perimeter of the disc shaped mixed micelles.

Another important point is the different dependence of the T_m of DPPC vesicles on R_{tot} depending on whether $[D_{tot}]$ at R_{sat} is above or below the cmc of the detergent (cf. Fig. 4B). It demonstrates that the presence of detergent micelles in equilibrium with vesicles below R_{sat} gives rise to a different solubilization behaviour. This is also indicated by the behaviour of ΔH versus R_{tot} under this condition (i.e., at [L] = 10.8 mM): For $R_{tot} > R_{sat}$, the enthalpy ΔH decreases continuously with increasing R_{tot}. However, the shape of the endotherms does not broaden as reported above for low [L] (Fig. 1F) but remains as a narrow peak at 37.0 °C until it vanishes completely in the flat baseline at $R_{tot} > R_{sat}$. The coexistence of detergent micelles and vesicles at $R_{tot} < R_{sat}$ (which is not the case for the measurements performed at low [L]) prevents the entropically unfavorable presence of detergent monomers in the water phase. As a result, the vesicles are not ruptured above R_{sat} into larger disc shaped mixed micelles as shown in Fig. 6, but solubilized in small micelles with consider-

ably higher detergent proportion. Such micelles give no detectable hs-DSC signal so that only the part of intact vesicles (which give a narrow peak) is observed by hs-DSC measurements at [L] = 10.8 mM (data not shown).

These findings are an indication that an entropic factor can contribute to the solubilization of DMPC and DPPC vesicles observed at detergent concentrations $[D_{tot}] < cmc$ at the membrane saturation concentration R_{sat}: As the detergent concentration $[D_{tf}]$ at the interface region of the vesicles (site 1) is limited mainly by the total surface of the vesicles and the detergent solubility in the aqueous bulk phase is restricted by $[D_m]$ ($[D_{tot}] = [D_{tf}] + [D_{hc}] + [D_m] < cmc$), the increasing entropy of the system above R_{sat} could provide an excess hydrophobic free energy

$$\Delta G_{ex} = RT \ln x'_w \tag{11}$$

(x'_w is the excess concentration ($x'_w > [D_m]$) of detergent monomers in the water phase in mole fraction units). This excess hydrophobic free energy could enable the detergent monomers to overcome the high free energy of transfer barrier $\mu^0_w - \mu^0_{hc}$ for DPPC to interact at site 2 of the vesicles at $R_{tot} \geq R_{sat}$.

Conclusions

The hs-DSC and FT-IR measurements performed in this work reveal striking differences of the interaction of detergents with DMPC and DPPC, respectively. They suggest two interaction sites of detergents in vesicles, one at the interface region and the other in the hydrocarbon chain region of the vesicles. The partition of detergents between these sites is mainly ruled by the intrinsic lateral pressure of the bilayer. As a consequence, for DMPC vesicles both sites are occupied by detergents at concentrations $R_{tot} < R_{sat}$ while for DPPC vesicles the detergents interact exclusively at one site below R_{sat}.

The considerable differences in the solubilization behaviour of vesicles observed at detergent concentrations thoroughly below the cmc from that observed above the cmc are likely caused by the presence of an additional entropic force in the former case.

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