Bile Salt Induced Solubilization of Synthetic Phosphatidylcholine Vesicles Studied by Isothermal Titration Calorimetry

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Bile Salt Induced Solubilization of Synthetic Phosphatidylcholine Vesicles Studied by Isothermal Titration Calorimetry

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Isothermal titration calorimetry (ITC) was used to investigate the interactions of bile salts with phosphatidylcholine vesicles. We determined the partition coefficients of the detergents sodium cholate (NaC) and sodium deoxycholate (NaDC) and the respective transfer enthalpies between pure water or 0.1 M aqueous salt solution and bilayers, consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC). Additionally, the vesicle-to-micelle transition was investigated for NaC/DPPC and NaDC/DPPC systems in water and 0.1 M NaCl. ITC was employed to determine the phase boundaries for the saturation and the complete solubilization of the vesicles by the bile salts enclosing the coexistence region of mixed vesicles and micelles. To study the influence of the alkyl chain length of the phospholipids on the phase behavior we also studied the NaDC/DMPC system. Saturated phosphatidylcholines are more easily transformed into micelles than those with unsaturated chains. In the region of low lipid concentrations we observed a departure from linearity of the phase boundaries, which was explained by the influence of the energy of end-caps as proposed by Roth et al. (*Langmuir* **2000**, *16*, 2052). The deviation was larger for systems in pure water compared to those in 0.1 M NaCl. The saturation concentrations of bilayers of DPPC and DMPC were much lower than those for unsaturated analogues. The saturation concentration increased with increasing salt content, and the coexistence range became wider. The ITC solubilization curves could be analyzed by applying the known values for partition coefficients and transfer enthalpies for the detergents to the different types of aggregates.

Introduction

Thermodynamic studies of mixtures of micelle-forming bile salts and bilayer-forming phosphatidylcholines (PC) are of general interest not only for the understanding of the self-assembly of supramolecular aggregates[1] but also for the design of new drug delivery systems such as liposomes[2−5] and mixed micelles.[6] Especially, the use of surfactants in pharmaceutical formulations is quite common as a tool to solubilize otherwise only slightly soluble drugs and to protect them from degradation.

Bile salts are physiological detergents and play an important role in the intestinal digestion and absorption of dietary lipids and cholesterol. These native detergents solubilize lipid vesicles by transforming them into mixed micelles. Bile salts, having a large, rigid, and planar hydrophobic moiety of a steroid nucleus with two or three hydroxyl groups (Figure 1) are a special group of biosurfactants, whose properties differ considerably from ordinary aliphatic surfactant molecules, the so-called "head−tail conical surfactants". They act as solubilizer and emulsifier for cholesterol and lipids in the intestine. Due to their structure and rigidity the aggregation properties are completely different compared to "normal" detergents.

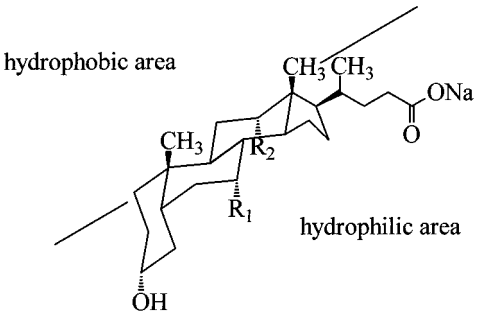

Figure 1. Chemical structure of bile salts with the hydrophobic and hydrophilic parts of the molecule: sodium cholate (NaC), R_1 = OH, R_2 = OH; sodium deoxycholate (NaDC), R_1 = H, R_2 = OH.

Mixed lipid/detergent systems play an important role for the investigation of membrane properties and functions[7,8] as well as the reconstitution and stabilization of membrane proteins.[9−13] In a first approximation, the

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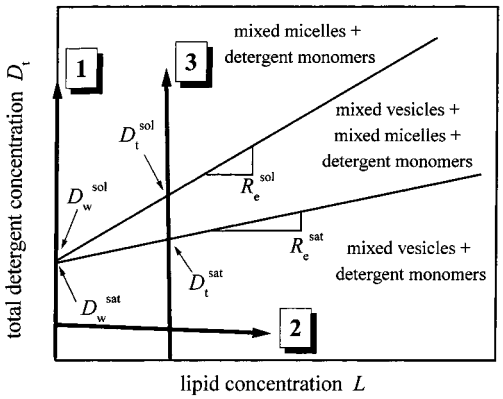

Figure 2. Schematic phase diagram of phospholipid/detergent mixtures (total detergent concentration D_t vs lipid concentration L) for the vesicle-to-micelle phase transition, indicating the phase boundaries D_t^{sat} and D_t^{sol}, the detergent/lipid ratios R_e^{sat} and R_e^{sol}, and the hypothetical detergent concentration in the presence of lipid D_w^{sat} and D_w^{sol} for the saturation and solubilization process.

vesicle-to-micelle transition can be described by the three-stage-model according to Lichtenberg.[1] By addition of detergent to lipid vesicles, the detergent molecules distribute between water and the lipid phase. The incorporation of detergent into the lipid bilayers yields an expansion of the vesicles until the saturation of the mixed bilayers is reached at a critical detergent/lipid ratio (R_e^{sat}). Below this critical concentration mixed vesicles coexist with detergent monomers. Above this phase boundary, lipid-rich mixed micelles are formed and coexist with mixed vesicles and detergent monomers. By further increase of the detergent concentration, all mixed vesicles are solubilized (R_e^{sol}). Only mixed micelles, detergent-rich and smaller in particle size, coexist with detergent monomers. In this transformation process, three different aggregation states occur as illustrated in Figure 2. This diagram is only valid for systems with high water content. With a decrease in water concentration a transformation into other aggregate structures can occur which is not relevant to the diluted systems treated here.

A number of different methods have been applied to study the interactions of detergents with lipid membranes,[14] the aggregation behavior in lipid/detergent mixtures, and the vesicle-to-micelle transition, e.g. light scattering,[15−19] small-angle neutron scattering,[20−24] electron microscopy,[25−28] calorimetry,[29−35] spectroscopy,[26,27,29,36−40] X-ray diffraction,[41,42] electron crystallography,[11] electron paramagnetic resonance,[43] and other biophysical techniques.[12,44]

In this study isothermal titration calorimetry (ITC)[7,35] was used to investigate the partitioning of NaC and NaDC molecules in PC bilayers and the solubilization of the PC vesicles by bile salts. Most published calorimetric investigations of the interaction of detergents with membranes were related to nonionic detergents. Heerklotz et al. studied the interactions in mixtures of the nonionic detergents $C_{12}EO_n$ (n = 3−8) and the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC).[33,34,45] Furthermore, systems with the nonionic detergent octyl-β-D-glucopyranoside (OG) were investigated by different groups. Keller et al. used different synthetic PC's and the native soy bean PC,[35] whereas Wenk et al. performed the experiments with POPC and mixtures with DMPC, the negatively charged phosphatidylglycerol, and cholesterol, respectively.[46] The investigations of Opatowski et al. dealt with egg-PC.[47,48] The interactions of lipid membranes with the octyl-β-thioglucopyranoside (OTG) were studied by Wenk and Seelig.[49] An overview of the partition behavior of a number of neutral detergents (oligo(ethylene oxide) alkyl ethers, alkyl glucosides, alkyl maltosides, short chain PC, Triton X-100 and X-114, and CHAPS) was presented by Heerklotz and Seelig.[50,51]

In this investigation we studied the interaction and solubilization of lipids by negatively charged bile salts using ITC. While numerous studies already exist on the solubilization of phospholipids by bile salts using other techniques, this is the first study that uses ITC to obtain partition coefficients, transfer enthalpies, and phase boundaries of the vesicles−micelle transition of saturated phospholipids. ITC offers the unique possibility to obtain these different thermodynamic quantities from one and

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the same experiment. Bile salts have a special molecular structure, and their aggregation behavior as well as their interaction with membranes is different from that of normal alkyl chain containing surfactants. With negatively charged surfactants it is expected that also the interaction with model membranes shows a dependence on ionic strength as is generally observed also for the micellization behavior. Indeed, different partitioning and solubilization properties of the bile salts are observed when they are dissolved in water compared to 0.1 M NaCl solution where electrostatic effects are partially screened. These differences can be understood by taking into account electrostatic effects and the preferences of the different amphiphiles for interfaces of different curvature.

Experimental Section

Materials. The bile salts sodium cholate (NaC) and sodium deoxycholate (NaDC) were purchased from Sigma (Deisenhofen, Germany). The purity of the bile salts was tested by mass spectrometry (Finnigan LCQ; Thermoquest, San Jose, CA). The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were products of Genzyme Pharmaceuticals, Sygena Facility (Liestal, Switzerland). The phospholipids were pure as checked by thin-layer chromatography.[52] Sodium chloride of pA grade was purchased from Merck (Darmstadt, Germany). All substances were used without further purification.

Sample Preparation. The bile salt solutions and the phospholipid dispersions were prepared by dissolving a certain amount in water or 0.1 M NaCl. All water used in this study was ultrapure as obtained from a Milli-Q system (Millipore; Molsheim, France). The lipid vesicle solutions were prepared by first vortexing a coarse lipid suspension for 5 min at a temperature above the respective T_m, followed by mild ultrasonication in a water bath at 60 °C for 20 min. The vesicle size was determined by dynamic light scattering using a Malvern Zetasizer 3 (Malvern Instruments, Herrenberg, Germany). The diameter of the vesicles was in a range of 80−120 nm. Vesicles of this size are usually to a large extent unilamellar. The solutions were degassed and the pH of all samples was adjusted to 7.4. All samples were freshly prepared.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry (DSC) measurements were performed with a VP-DSC scanning calorimeter (MicroCal, Inc., Northampton, MA). The heating rate was 1 °C·min^{-1}. Heating curves were measured in the temperature interval from 5 to 95 °C, but only the temperature range in which phase transitions are observed is shown.

Isothermal Titration Calorimetry (ITC). The ITC experiments were performed either with a MicroCal OMEGA titration calorimeter or an MCS ITC unit (MicroCal, Inc., Northampton, MA). The calorimeter sample cell (OMEGA, 1.3684 mL; MCS, 1.3351 mL cell volume) was filled with the phospholipid dispersion or the bile salt solution (depending on the titration experiment; see below), and the titration syringe (250 μL) was loaded with the reaction partner outside of the instrument. During the experiment the syringe rotated at ∼400 rpm, and the volume of the syringe was injected into the cell in 5−10 μL aliquots. The reference cell was filled with buffer. The concentrations were chosen in such a way that the processes of partitioning and solubilization occurred during the experiments (see below). The lipid/detergent interaction was studied in the liquid-crystalline phase of the membranes, i.e., at temperatures above the main phase transition of the lipids under investigation. The DPPC measurements were performed at 60 °C, and the DMPC measurements at 30 °C. The experimental data were analyzed using the ORIGIN software (MicroCal).

Partitioning Experiments. For the partitioning experiments (see arrow no. 2 in Figure 2), a highly concentrated phospholipid dispersion was titrated into the sample cell containing the bile salt solution with a concentration below the critical micellization concentration (cmc). The partitioning of NaC or NaDC in DPPC vesicles was studied in water and 0.1 M NaCl at pH 7.4. We used the same salt concentration to be able to compare the partitioning results with the micellization process studied before.[7]

DPPC dispersions with a concentration of 20 mM were titrated into a 10 mM NaC or a 4 mM NaDC solution in water and into a 4 mM NaC or a 1 mM NaDC solution in 0.1 M NaCl, respectively. Three to five individual measurements were performed for each system. The heats of dilution were obtained by titrating the lipid dispersion into pure water or 0.1 M NaCl and subtracted from the molar titration heats.

The nonlinear least-squares fits of the experimental data were performed using the SCIENTIST for windows software (MicroMath Scientific Software, Inc., Salt Lake City, UT) as described before.[7]

Solubilization Experiments. The solubilization experiments (see arrow no. 3 in Figure 2) were performed with a phospholipid dispersion in the cell. A bile salt solution with a high concentration well above the cmc was added. The solubilization of DPPC with both bile salts, NaC and NaDC, was investigated in water and 0.1 M NaCl, respectively. The NaC solutions had a concentration of 150 mM in water and 100 mM in 0.1 M NaCl; the NaDC solutions were titrated into the calorimeter cell with a concentration of 100 mM in water and 50 mM in 0.1 M NaCl. For the DMPC experiments the bile salt NaDC with a concentration of 150 mM in water was used. To test the reproducibility three to five individual experiments were carried out.

Results and Discussion

Partitioning of Bile Salts into DPPC Bilayers. ITC is a suitable method to study quantitatively the detergent partitioning of the bile salts NaC and NaDC into PC lipid membranes. A surfactant dissolved in the aqueous phase at a concentration below its critical micellization concentration (cmc) will partition into the membranes without disrupting the bilayer (see arrow no. 2 in Figure 2). With increasing amount of detergent accumulated in the membrane, the physical properties of the membrane will gradually change. Therefore, it is to be expected that the partitioning coefficient will depend on the concentration of the surfactant already present in the bilayer. Particularly with surfactants such as bile salts, the mixing properties in the bilayer will be highly nonideal. In addition, charge effects have to be considered. Incorporation of negatively charged bile salt molecules into the bilayer will lead to a negatively charged membrane surface, which in turn decreases the local bile salt concentration in the vicinity of the surface. The electrostatic effects can be incorporated into a partitioning model, which takes these effects into account.[35,50,53] The principle advantage of ITC over other methods is that with ITC not only the partition coefficient P but also the heat of transfer ΔH^T of detergent molecules from water to bilayers can be determined.

For partitioning experiments a PC vesicle dispersion in the injection syringe was titrated into the calorimeter sample cell containing the bile salt solution with a concentration below the cmc (see arrow no. 2 in Figure 2). Figure 3 shows experiments of the partitioning of NaC (10 mM) and NaDC (4 mM) respectively into DPPC (20 mM) membranes in water at $T = 60$ °C. The lipid vesicles were added in small aliquots of 5 μL. A total of 50 injection steps were performed.

From the heat flow vs time titration curves (Figure 3A′,A″) it is obvious that each lipid vesicle injection leads to a partitioning of detergent into the membrane and produces an exothermic heat of reaction. The detergent concentration in the calorimeter cell decreases slightly with each injection due to the dilution of the bile salt solution with the lipid dispersion, whereas the lipid

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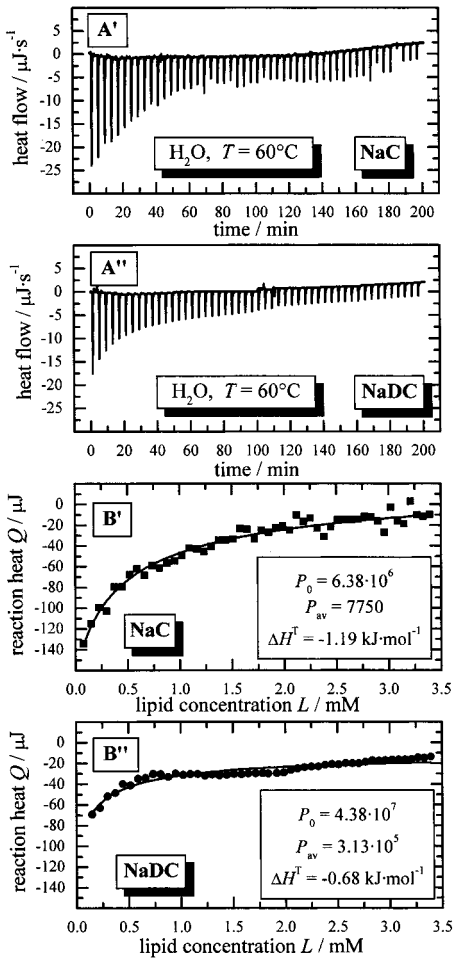

Figure 3. Partition experiment: Titration of a DPPC vesicle dispersion (20 mM) into a NaC (10 mM) or a NaDC (4 mM) solution in H_2O with 50 × 5 μL injections at $T = 60$ °C. Key: (A) calorimetric traces; (B) change of reaction heat ΔQ of each injection vs lipid concentration L in the cell.

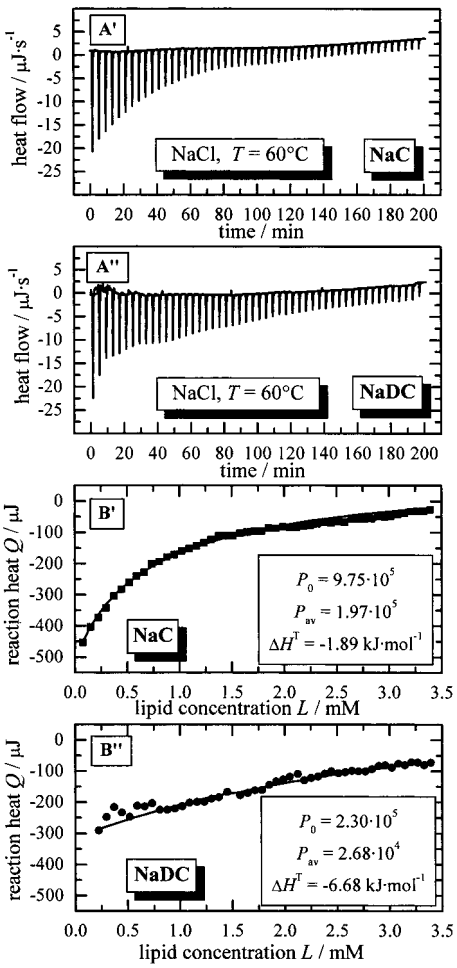

Figure 4. Partition experiment: Titration of a DPPC vesicle dispersion (20 mM) into a NaC (4 mM) or a NaDC (1 mM) solution in 0.1 M NaCl with 50 × 5 μL injections at $T = 60$ °C. Key: (A) calorimetric traces; (B) change of reaction heat ΔQ of each injection vs lipid concentration L in the cell.

concentration increases up to approximately 3.5 mM. The molar lipid/detergent ratio reached in the sample cell after 50 injections of 5 μL is ~0.4 for NaC and ~1.0 for NaDC. As the lipid concentration in the sample cell increases, the reaction enthalpies decrease in magnitude until the reaction peaks become quite small and nearly constant. This is due to the fact that less and less detergent in the cell is available for binding and incorporation. The reaction heat changes ΔQ of each injection as a function of the lipid concentration L in the cell are determined by integrating the reaction peaks (Figure 3B′,B″).

Figure 4 shows the partition experiments of NaC (4 mM) and NaDC (1 mM) respectively into DPPC (20 mM) membranes in aqueous 0.1 M NaCl solution at $T = 60$ °C. In this case, the molar lipid/detergent ratio reached in the sample cell at the end of the titration experiment was ~1.0 for NaC and ~4.0 for NaDC.

For the determination of the partition coefficients from the titration curves we used a partition coefficient P in mole fraction units, the calculation procedure incorporating the electrostatic effects due to the charging of the bilayers[53−56] by the incorporated bile salts is described in the Appendix. The thermodynamic parameters for the partitioning of NaC and NaDC into DPPC bilayers in water and in aqueous 0.1 M NaCl are summarized in Table 1 and compared with the values for the micellization.

Table 1. Thermodynamic Parameters Obtained from the Partition Experiments in NaC/DPPC and NaDC/DPPC Systems in Water and 0.1 M NaCl and for the Micellization[7] at $T = 60$ °C

	NaC/DPPC		NaDC/DPPC	
param	H_2O	0.1 M NaCl	H_2O	0.1 M NaCl
P_0	6.4×10^6	9.8×10^5	4.4×10^7	2.3×10^5
P_{av}	7750	2.0×10^5	3.1×10^5	2.7×10^4
ΔH^T/kJ·mol^{-1}	−1.2	−1.9	−0.7	−6.7
ΔG^T_0/kJ·mol^{-1}	−43.4	−38.2	−48.7	−34.2
ΔG^T_{av}/kJ·mol^{-1}	−24.8	−33.8	−35.0	−28.3
$T\Delta S^T_0$/kJ·mol^{-1}	42.2	36.3	48.1	27.5
$T\Delta S^T_{av}$/kJ·mol^{-1}	23.6	31.9	34.4	21.6
ΔG^T_{micell}/kJ·mol^{-1}	~−22	~−23	~−20	~−26
ΔH^T_{micell}/kJ·mol^{-1}	~−8	~−7	~−13	~−13
$T\Delta S^T_{micell}$/kJ·mol^{-1}	~16	~16	~7	~13

The values of the transfer enthalpies ΔH^T are negative for the transfer of bile salt molecules from the aqueous phase into DPPC vesicles. The micellization enthalpies obtained for NaC or NaDC respectively in pure water or 0.1 M NaCl are also negative at all temperatures above $T = 30$ °C.[7] At the experimental temperature of 60 °C

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used in our partition experiments, the values for the transfer enthalpy from water to a surfactant micelle are ∼−8 and ∼−13 kJ·mol^{-1} for NaC and NaDC, respectively, in water and ∼−7 and −13 kJ·mol^{-1}, respectively, in 0.1 M NaCl. These values are much more negative than the transfer enthalpies ΔH^T from water to a liquid-crystalline DPPC bilayer. When the Gibbs free energy changes ΔG^T are compared, one finds values in the range of −20 to −26 kJ·mol^{-1} for transfer from water to a micelle but much higher values for transfer to PC vesicles. In addition, the ΔG^T values depend on the amount of bile salt already in the bilayer; i.e., they decrease with higher surfactant concentration in the bilayer. There are two effects that are causing this decrease. The first one is the buildup of negative charge at the bilayer surface, which lowers the bile salt concentration in the vicinity of the bilayer. This can be calculated using the Gouy−Chapman-theory with eqs A6−A9 (see Appendix). The apparent partition coefficient therefore decreases according to eq A6; the intrinsic partition coefficient, however, is a constant. The second effect is the nonideal mixing behavior of the surfactant with the lipid (see eq A6). This will also decrease the partition coefficient and has also been observed for nonionic surfactants.[35] In the simulations it turned out that the nonideal mixing effect is of minor importance in getting good fits; the nonideality values ρ were between 0 and 1000 J mol^{-1}.

Addition of 0.1 M NaCl should decrease the partition coefficient, because in salt-containing solutions the charges are screened to a large effect and electrostatic effects are therefore reduced. This is indeed observed for NaC, as the higher values of P_{av} and larger ΔG^T_{av} for 0.1 M NaCl solution in Table 1 clearly show. The P_0 value should not be affected, because the screening effects at infinite dilution of the bile salt in the bilayer vanish. The observed differences for P_0 and ΔG^T_0 are probably within the margin of error in the simulations.

However, for NaDC, the effect of NaCl is just the opposite as expected (see Table 1). One possibility for this observation is the difficulty of fitting the titration curve of NaDC in 0.1 M NaCl (Figure 4B″). The experimental titration curves show marked differences for the two systems NaC/DPPC and the NaDC/DPPC in water and in 0.1 M NaCl. Whereas the titration curves for NaC (Figures 3B′ and 4B′) are continuous and the calculated curves fit much better, this is different for NaDC (Figures 3B″ and 4B″). The curves seem to consist of an initial part with a larger slope and a subsequent part with a much weaker slope. The fits of the calculated curves using just one single partitioning mechanism are therefore not as good as for NaC. The incorporation mechanism for NaDC into DPPC membranes may be different for the less hydrophilic dihydroxy bile salt NaDC compared to NaC. We also observed much larger differences in the solubilization behavior of NaDC in water and 0.1 M NaCl (see below) compared to NaC, which are not easily explained.

The ΔH^T values normally show a strong temperature dependence caused by a change in molar heat capacity when the hydrophobic surface exposed to water is decreased. For the micellization of bile salts ΔH^T is zero around 300 K and the ΔC_p values for micellization are between −260 and −360 J·K mol^{-1} at 298 K.[7] For the partitioning we find a relatively low ΔH^T value at 60 °C, much smaller than for the micellization process (see Table 1). This is either due to a complete shift of the ΔH^T vs T curve, i.e., a much higher temperature where ΔH^T is zero, or to a much lower ΔC_p value for the partitioning into lipid bilayers. Due to the relatively high main phase transition of DPPC (41.5 °C) the partitioning experiments could not be performed over the same temperature range as the demicellization experiments. Therefore, the typical change of sign of the transfer enthalpies between lower and higher temperatures was not investigated in this case.[35] The question therefore remains open whether the ΔC_p values are lower for the partitioning compared to the micellization. Ollila and Slotte recently published some ΔH^T values for bile salt partitioning into lipid bilayers at 25 °C. They reported values of 11 and 9 kJ·mol^{-1} for the partitioning of cholate and deoxycholate, respectively, into egg-PC bilayers in 140 mM salt.[57] For ΔC_p for partitioning into egg-PC bilayers they measured a value of −433 J·K mol^{-1}, i.e., a slightly larger ΔC_p value for partitioning than for the micellization process. This is at variance with our recently measured data for partitioning of NaC and NaDC into POPC vesicles in pure water in the temperature range between 20 and 60 °C. We found a much lower ΔC_p value of −50 to −100 J·K mol^{-1} (K. Beyer, A. Hildebrand, P. Garidel, R. Neubert, and A. Blume, unpublished results). Addition of 100 mM NaCl increased ΔC_p only slightly in the case of NaC, while again for NaDC the behavior was quite different, ΔC_p increasing to −300 J·K mol^{-1}. From these preliminary results we conclude that the change in exposed hydrophobic surface area is actually less for the transfer of bile salt monomers into a bilayer compared to a micellar aggregate, i.e., that in the bilayer more of the hydrophobic surface of the bile salt molecules is exposed to water than in a micellar aggregate. However, it is also possible that the low ΔC_p values are partly due to the exposition of hydrophobic surfaces in the lipid molecules when the bile salts are incorporated and perturb the bilayer. The recent study of partitioning of alcohols of different chain lengths by Rowe et al.[58] showed no systematic increase of ΔC_p with chain length of the alcohol. This was also interpreted as being caused by possible changes in lipid interactions with water in the interfacial region. The interpretation given in this paper agrees with our notions. However, a separation of the ΔC_p contributions arising from the lipid and those from the bile salt is not possible.

The ΔG^T values are normally only slightly temperature dependent. Taking an endothermic ΔH^T value at 25 °C, this would lead to the conclusion that the $T\Delta S^T$ term is overcompensating the positive ΔH^T value at room temperature; i.e., the increase in entropy by transferring a bile salt molecule from water to a bilayer is much larger than for the transfer to a micelle.

Solubilization of Phosphatidylcholine Bilayers by Bile Salts. *Solubilization of DPPC in Water*. The arrow no. 3 in Figure 2 describes schematically the solubilization experiment and solubilization protocol used in this study. The surfactant concentration where membrane solubilization occurs is dependent on the total concentration of lipid vesicles, as the scheme in Figure 2 shows. For higher lipid concentrations a surfactant concentration higher than the cmc is needed.

The composition of the mixed aggregates is described by the effective detergent-to-lipid ratio R_e:

$$R_e = \frac{D_b}{L} \tag{1}$$

Here L is the lipid concentration and D_b the detergent concentration in the mixed aggregates.

(57) Ollila, F.; Slotte, J. P. *Langmuir* **2001**, *17*, 2835

(58) Rowe, E. S.; Zhang, F.; Leung, T. W.; Parr, J. S.; Guy, P. T. *Biochemistry* **1998**, *37*, 2430.

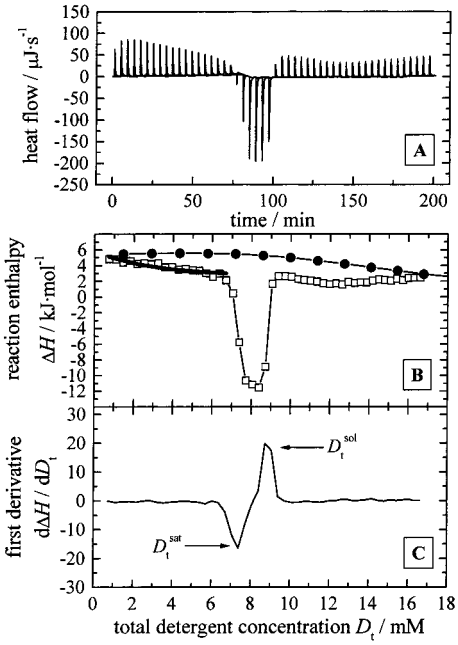

Figure 5. Solubilization experiment: Titration of a micellar NaC solution (100 mM) into a DPPC dispersion (6 mM) in 0.1 M NaCl with 50 × 5 μL injections at T = 60 °C. Key: (A) calorimetric traces; (B) normalized titration heat Q vs total detergent concentration in the cell D_t; (C) first derivative of curve B. In (B) the reference curve for demicellization of NaC is included (−●−).[7] The full line in (B) was calculated using the partitioning model described in the text with a value for the transfer enthalpy $\Delta H_{\text{detergent}}^{\text{mic}\rightarrow\text{bil}}$ of 5.4 kJ·mol^{-1}.

With the total detergent concentration $D_t = D_w + D_b$, where D_w is the detergent concentration in the aqueous phase, eq 1 can be expressed by

$$D_t^{\#} = R_e^{\#} \cdot L + D_w^{\#} \qquad (2)$$

where "#" means "sat" and "sol", respectively.[59] The effective detergent/lipid ratios R_e for the saturation and the solubilization of the vesicles are determined from the slopes of the straight lines indicating the phase boundaries which are obtained from linear least-squares fits of the experimental data for D_t^{sat} and D_t^{sol} (see Figure 2). The intercepts, D_w^{sat} and D_w^{sol}, established by extrapolation of the phase boundaries to a lipid concentration L = 0 mM, correspond to the hypothetical detergent concentration of aggregation in the presence of phospholipid vesicles and are so the minimal detergent concentration required for the solubilization of lipid membranes.

To investigate the solubilization of lipid vesicles a concentrated bile salt solution is added in small aliquots (typically 5−10 μL) to the sample cell containing the phospholipid dispersion of a defined concentration. Thus, the solubilization protocol is based on the injection of detergent above the cmc into the vesicle suspension. Figure 5 shows a solubilization experiment with a 6 mM DPPC vesicle dispersion and a 100 mM NaC solution, both in 0.1 M NaCl, at 60 °C. A total of 50 injection steps of 5 μL bile salt solution were added.

The first 19 injections show a continuous, endothermic course in heat flow vs time (Figure 5A). In this range the detergent concentration in the cell remains lower than the critical concentration for membrane solubilization. In this concentration range the peaks are caused by the demicellization process of the pure micelles followed by the incorporation of the monomers into the lipid membrane. The observed reaction enthalpy in Figure 5A is the sum of the demicellization enthalpy and the partitioning enthalpy of the detergent monomers from water to the lipid membrane, the latter multiplied by the fraction of bile salts partitioning into the bilayers. At very low detergent concentration the reaction enthalpy observed in Figure 5B is initially 5 kJ·mol^{-1} and decreases linearly to 3 kJ·mol^{-1} at the onset of bilayer solubilization. The reference curve for the demicellization of NaC at the same temperature is included in Figure 5B. From the difference of the two curves the transfer enthalpy of detergent from water into a lipid bilayer can be calculated once the partition coefficient is known. This was determined in a separate experiment (see above) to $P_0 = \sim 1 \times 10^6$ with $\Delta H^T = -1.9$ kJ·mol^{-1} (see Table 1). Taking these two values and a demicellization enthalpy of +7 kJ·mol^{-1}, one calculates values around 5.5 kJ·mol^{-1} for the initial part of the curve shown in Figure 5B. This is in very good agreement with the experimental values. The solid curve through the initial points stems from a direct simulation using the partition model described above, except that now the enthalpy consists of the sum of the demicellization process and the partitioning enthalpy, i.e., the transfer enthalpy $\Delta H_{\text{detergent}}^{\text{mic}\rightarrow\text{bilayer}}$. This direct simulation gave a partition coefficient P_0 of 7.8 × 10^5, i.e., very close to the one shown in Table 1, and a transfer enthalpy $\Delta H_{\text{detergent}}^{\text{mic}\rightarrow\text{bilayer}}$ of 5.4 kJ·mol^{-1}, also very close to the difference in the values ΔH^Tmicelle and ΔH^T shown in Table 1 (5 kJ·mol^{-1}).

Further addition of 5 μL aliquots of NaC solution then yields a sudden change of the enthalpic effects. Large exothermic reaction heats are observed until peak no. 25 is reached. At the beginning of this range the free concentration of detergent in the cell reaches a critical concentration at which the bilayer membrane become saturated with detergent molecules and is unable to incorporate more detergent in the bilayer. At this concentration D_t^{sat} the detergent-saturated mixed vesicles of the composition R_e^{sat} start to disintegrate and form mixed lipid detergent micelles of the composition R_e^{sol}. With increasing amount of detergent concentration this equilibrium is shifted toward the mixed micelle structure. As can be seen from Figure 5B, the observed reaction enthalpies are constant in this range. This is in accordance with the phase rule approximation, which predicts that the reaction enthalpies are constant over the whole coexistence range.[34] The observed value for the reaction heat in this range is ∼−11 kJ·mol^{-1}. It arises from a transfer of detergent and lipid molecules from mixed bilayers to mixed micelles. Using the expression eq 18 of ref 34

$$\Delta H = -\frac{x_{sat}(1-x_{sol})}{x_{sol}-x_{sat}}\Delta H_{\text{detergent}}^{\text{mic}\rightarrow\text{bilayer}} + \frac{(1-x_{sol})(1-x_{sat})}{x_{sol}-x_{sat}}\Delta H_{\text{lipid}}^{\text{bil}\rightarrow\text{micelle}} + \Delta H_{\text{detergent}}^{\text{mic}\rightarrow\text{mixmic}} \qquad (3)$$

with

$$x_{sat} = \frac{R_e^{sat}}{1+R_e^{sat}} \quad x_{sol} = \frac{R_e^{sol}}{1+R_e^{sol}}$$

one can calculate the observed reaction enthalpies in this region. With a value for $\Delta H_{\text{lipid}}^{\text{mic}\rightarrow\text{bil}}$ of 5 kJ·mol^{-1} and x_{sat} and x_{sol} values of 0.16 and 0.225, respectively, the first term alone already amounts to −10 kJ·mol^{-1}. This means

(59) Lichtenberg, D. *Biochim. Biophys. Acta* **1985**, *821*, 470.

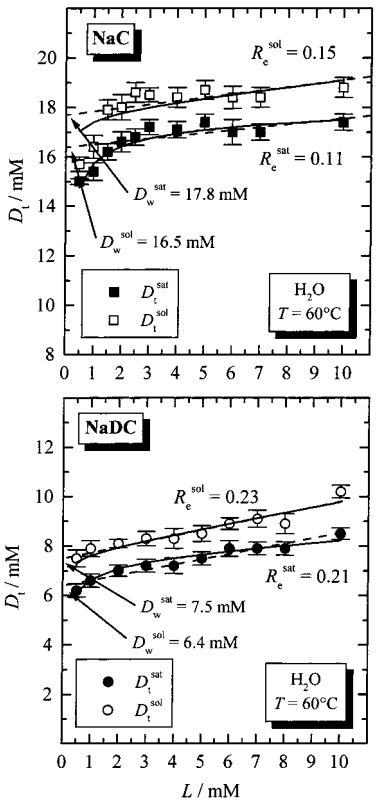

Figure 6. Phase diagrams for the vesicle-to-micelle transition in NaC/DPPC and NaDC/DPPC systems in H_2O at $T = 60$ °C: D_t^{sat} filled, D_t^{sol} open symbols. Dashed lines represent R_e^{sat} and R_e^{sol} values obtained from linear least-square fits of the experimental data (2−10 mM for NaC/DPPC), with D_w^{sat} and D_w^{sol} values from the extrapolation to $L = 0$ mM. Full lines were calculated using the model of Roth et al.[59] with the following parameters: NaC/DPPC, $D_w = 18$ mM, $R_e^{sat} = 0.022$, $R_e^{sol} = 0.13$, $2\epsilon = 22\ kT$; NaDC/DPPC, $D_w = 8$ mM, $R_e^{sat} = 0.082$, $R_e^{sol} = 0.211$, $2\epsilon = 21.2\ kT$.

that the transfer enthalpies in the second and third term, $\Delta H_{lipid}^{bil\rightarrow micelle}$ and $\Delta H_{detergent}^{mic\rightarrow mixmic}$ are either small or compensate, the first notion being more likely, because the term $\Delta H_{detergent}^{mic\rightarrow mixmic}$ should already be zero for ideal mixing and because the prefactor for the second term is 5-fold higher than for the first term.

Further addition of bile salt induces a second drastic change, and smaller endothermic peaks are observed at the end of the titration. During the titration experiment the second phase boundary detected corresponds to the complete disintegration and transformation of mixed vesicles to mixed micelles. Above this concentration only mixed micelles occur in the sample in coexistence with detergent monomers. The reaction enthalpy observed in this range is therefore the heat measured for the equilibration of injected pure micelles with mixed micelles in the sample cell. The small endothermic effects are caused by a nonzero $\Delta H_{detergent}^{mic\rightarrow mixmic}$; i.e., mixing in the micelles is nonideal.

Integration of the peaks yields the molar titration heat Q as a function of the total detergent concentration D_t in the sample cell (Figure 5B). The two break points in the titration curve correspond to points on the phase boundaries of the coexistence range. The specific values for saturation, D_t^{sat}, and solubilization D_t^{sol}, i.e., points on the phase boundaries, are usually determined from the extreme values of the first derivative curve (Figure 5C).

The resulting phase diagrams for NaC/DPPC and NaDC/DPPC mixtures in water are shown in Figure 6.

Table 2. Slopes of the Saturation (R_e^{sat}) and Solubilization (R_e^{sol}) Lines and Equilibrium Bile Salt Concentrations $D_w^{\#}$ in NaC and NaDC/Phosphatidylcholine Systems in Water and 0.1 M NaCl at $T = 60$ or 30 °C[a]

	NaC/DPPC		NaDC/DPPC		
param	H_2O	0.1 M NaCl	H_2O	0.1 M NaCl	NaDC/DMPC (H_2O)
T/°C	60	60	60	60	30
R_e^{sat}	0.11 (0.022)	0.19	0.21 (0.082)	0.20	0.064
R_e^{sol}	0.15 (0.13)	0.29	0.23 (0.211)	0.39	0.065
D_w^{sat}/mM	16.5 (18)	5.6	6.4 (8)	1.4	3.3
D_w^{sol}/mM	17.8 (18)	6.6	7.5 (8)	1.7	4.4

[a] Values in parentheses refer to results from model calculations using the approach of Roth et al.[59]

The experiments with the DPPC systems were carried out at $T = 60$ °C, well above the main phase transition temperature T_m of DPPC (41.4 °C[1]). Due to the relatively high phase transition temperature, the temperature dependence of the solubilization process was not investigated.

The coexistence range seems to be quite narrow for both bile salts; it has only a width of about 1−2 mM (see Figure 6), and the phase boundaries have only a small slope. The R_e and D_w values are summarized in Table 2.

There is a clear difference between the trihydroxy bile salt NaC and NaDC with only two hydroxyl groups per molecule in their solubilization behavior. A considerably smaller detergent concentration is necessary for the solubilization of DPPC membranes with the more hydrophobic NaDC than with NaC. This is in agreement with the lower cmc of NaDC (19.5 mM at 60 °C) compared to NaDC (10.5 mM at 60 °C).

It can be seen from Figure 6 that D_w^{sat} and D_w^{sol} differ by approximately 1 mM and do not have the same value as expected according to the basic model of Lichtenberg.[1,59] In addition, the D_t^{sat} and D_t^{sol} values do not lie on a straight line. This observation has already been made before for the solubilization using nonionic surfactants, such as octylglucoside (OG). Linearity of the phase diagrams is only observed for lipid concentrations higher than 1.5 mM for NaC in water and 1 mM for NaDC in water (see Figure 6). Below this concentration the equilibrium aggregation state seems to be concentration dependent. A curvature of the phase boundaries becomes obvious at low lipid concentrations. Unfortunately, this phenomenon could not be followed to lipid concentrations lower than 0.5 mM, due to the detection limit of ITC method. The original 3-stage model implies that the composition of the coexisting aggregates does not change throughout the whole coexistence range. Because this is not experimentally observed in many cases, Lichtenberg and co-workers (Roth et al.[60]) have reevaluated the thermodynamics of solubilization at low lipid concentration taking into account the finite size of the mixed micelles and the energetic effects of end-caps of cylindrical micelles. This model can explain the nonlinear decrease of D_t^{sat} and D_t^{sol} at low lipid concentrations. We have simulated the phase boundaries of NaC and NaDC in water using this model and found considerable better agreement with the experimental data. The calculated curves are shown in Figure 6 as full lines, and the parameters are shown in the figure legend. As published by Roth et al.,[60] we found that the scission energy 2ϵ amounts to approximately 20−22 kT. The R_e^{sat} values found with the extended model are much

(60) Roth, Y.; Opatowski, E.; Lichtenberg, D.; Kozlov, M. M. *Langmuir* **2000**, *16*, 2052.

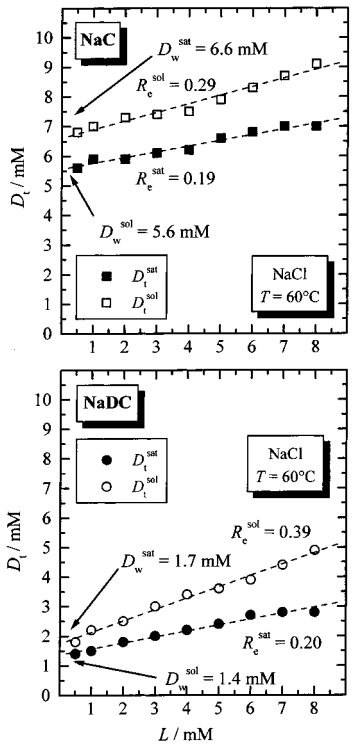

Figure 7. Phase diagrams for the vesicle-to-micelle transition in NaC/DPPC and NaDC/DPPC systems in 0.1 M NaCl solution at $T = 60$ °C. Phase boundaries are shown with the D_t^{sat} (filled) and D_t^{sol} (open) values for DPPC concentrations between 0.5 and 10 mM. The R_e^{sat} and R_e^{sol} values were obtained from linear least-squares fits of the experimental data, with the D_w^{sat} and D_w^{sol} values from the extrapolation to $L = 0$ mM.

smaller than determined from a linear least-squares fit of points in the higher concentration region. The R_e^{sol} values calculated from the model agree more or less with those from the linear least-squares fit (see Table 2).

The comparison with the R_e values of nonionic detergents shows large differences. For both NaC and NaDC, R_e values are much smaller and the difference between R_e^{sat} and R_e^{sol} is also very small. For OG/DMPC systems studied by Keller et al.[35] the following values were found: $R_e^{sat} = 1.66$ and $R_e^{sol} = 2.03$ at 70 °C. For $C_{12}EO_8$/POPC systems investigated by Heerklotz et al.,[34] $R_e^{sat} = 1.45$ and $R_e^{sol} = 5.0$ at 25 °C. For NaC and NaDC, however, the R_e values are in the range from 0.03 to 0.2. This means that the saturation limit is reached at a very low effective bile salt concentration in the bilayer, a much lower concentration than needed with alkylglucosides, for instance.

Solubilization of DPPC in 0.1 M NaCl. To investigate the influence of the ionic strength of the solubilization process, the experiments were also performed in 0.1 M NaCl solution (Figure 7) analogous to the measurements in water. Increasing the ionic strength reduces the electrostatic repulsion of the negatively charged molecules, favoring the aggregation process and the stabilization of the micelles. This phenomenon is well-known; for bile salts it was found that the cmc at 60 °C for NaC in water and in 0.1 M NaCl at pH 7.4 is 19.5 and 14.2 mM, respectively, whereas for NaDC the cmc is 10.5 mM in water and reduced 4.1 mM in 0.1 M NaCl.[7,61,62]

In 0.1 M NaCl the required detergent concentration for the solubilization is likewise reduced compared to solubilization in water. The D_w values decrease in the same way as the decrease in the cmc, i.e., to ~6 mM for NaC and 1.5 mM for NaDC (see Table 2). The $D_t^{\#}$ values now show an almost linear dependence on the lipid concentration, justifying the application of the simple linear relationship to obtain $R_e^{\#}$ values (Figure 7). The R_e^{sat} values are ~0.20 for both bile salts, but the R_e^{sol} values are significantly higher, namely 0.29 for NaC/DPPC and 0.39 for NaDC/DPPC. The remarkable feature for the systems in 0.1 M NaCl is therefore the broadening of the coexistence range, particularly for NaDC.

In experiments using light scattering and turbidity measurements to determine the dependence of the phase boundaries of NaC on electrolyte concentration Meyuhas et al.[62] found a decrease of D_w with increasing salt concentration from ~11 mM in pure water to ~5 mM in 0.1 M NaCl solution for the solubilization of egg-PC with NaC at 25 °C. However, the R_e^{sat} and R_e^{sol} values of ~0.3 and ~0.6, respectively, did not show any particular dependence on salt concentration. Compared to our results on DPPC solubilization with NaC we find a similar decrease of D_w with salt concentration but also a change of the $R_e^{\#}$ values. These differences are either due to the difference in temperature (60 °C vs 25 °C) but are probably more caused by the difference in chemical structure of the chains. Egg-PC has a high percentage of unsaturated C_{18} acyl chains, whereas DPPC has saturated C_{16} acyl chains. The breakdown of a lipid bilayer and the formation of mixed micelles will surely depend on the nature and length of the chains. Thus, it appears as if saturated PC are more susceptible to transformation into mixed micelles than PCs with unsaturated chains, which prefer surfaces with negative spontaneous curvature. Unpublished experiments for the solubilization of soy bean PC with unsaturated chains with NaC and NaDC performed in our own laboratory using ITC confirm this observation.

In ITC experiments, the determination of the phase boundaries is done by injecting aliquots of a surfactant solution above the cmc into the vesicle suspension. The whole experiment is finished within ~3 h. The question arises whether kinetic effects can play a role. In stopped-flow kinetic experiments with octylglucoside (OG) and saturated PCs we could show that the half-time for OG flip-flop is in the second range (Gutewort and Blume, to be published). So, for a nonionic detergent with a short alkyl chain the flip-flop occurs well within the waiting times between our injections. However, when the negatively charged bile salts NaC and NaDC are used, this flip-flop might be slower, because a charge bile salt molecule has to be transported through the bilayer. In some of our titration curves we had some hints that the peaks became broader at the base, i.e., their half width significantly larger than the response time of the instrument.

We wanted to pursue this problem further and performed titration experiments with higher resolution. In Figure 8 a solubilization experiment of a 2 mM DPPC vesicle dispersion with 50 mM NaDC solution in 0.1 M NaCl at 60 °C is shown for different experimental protocols. For the first experiment (Figure 8A*,B*) the NaDC solution was injected into the sample cell with 50 steps of 5 μL each. In a second experiment (see Figure 8A**,B**) we then studied in more detail what happened close to the point of detergent saturation of the bilayers (the 10 marked injections in the box of Figure 8A*). For this, the bile salt solution was added in 1 μL steps. A discontinuity of the titration heat could be observed for the injections 5−8 in Figure 8A**. The peak height suddenly decreased for several injections and then increased again below the

(61) Vinson, P. K.; Talmon, Y.; Walter, A. *Biophys. J.* **1989**, *56*, 669.
(62) Meyuhas, D.; Bor, A.; Pinchuk, I.; Kaplun, A.; Talmon, Y.; Kozlov, M. M.; Lichtenberg, D. *J. Colloid Interface Sci.* **1997**, *188*, 351.

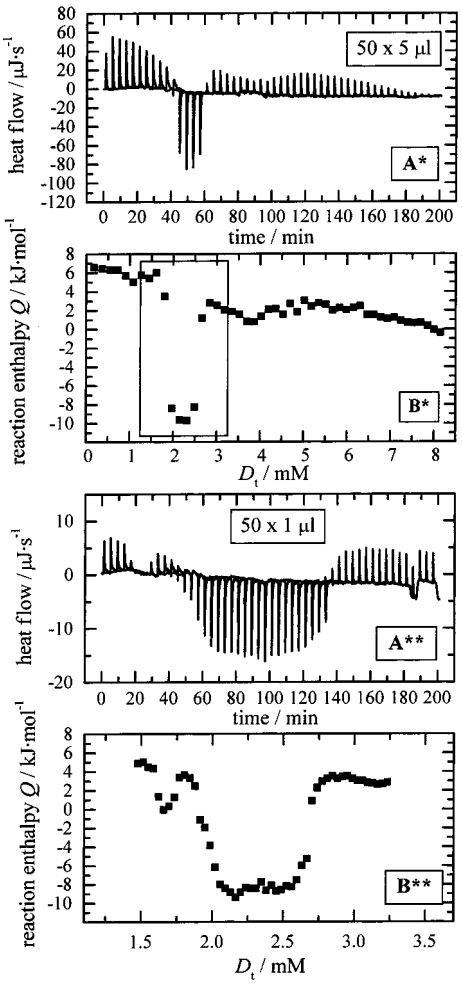

Figure 8. Solubilization experiment: titration of a micellar NaDC solution (50 mM) into a DPPC dispersion (2 mM) in 0.1 M NaCl with 50 × 5 μL injections (asterisk) and 50 × 1 μL injections (double asterisk) at $T = 60$ °C: (A) calorimetric traces; (B) normalized titration heat Q vs total detergent concentration in the cell D_t. From the titration with only 1 μL injections, i.e., with a higher resolution, more detailed information about the onset and offset of the phase transition was obtained.

saturation limit of the bilayers. This "gap" is plainly visible in the graph of the molar titration heat ΔH as a function of the total detergent concentration D_t (Figure 8B**). This effect was only observed for NaDC/DPPC mixtures in 0.1 M NaCl; in the other systems sometimes only indications of reduced kinetics, i.e., a broadening of the peaks prior to saturation, were visible. The "gap" in the peaks shown in Figure 8 could probably be caused by a saturation of the outer monolayer with NaDC leading to a decrease of the heat effects. Further increase of the surfactant concentration then renders the bilayers more permeable, and the bile salt molecules can flip-flop to the inner monolayers of the vesicles and the heat effect increases again. Whether for the other systems this process is faster or occurs only very close to the saturation limit is unclear at the moment.

The kinetics of bile salt flip-flop after the incorporation of bile salts molecules into the bilayer and thus the possible intermediate asymmetrical distribution of bile salt in the vesicles is still under debate. Data for the kinetics of transbilayer movement of bile salts in unilamellar egg PC liposomes have been reported by Cabral et al.[63] and Donovan and Jackson.[64] The flip-flop rates of bile salts, ^{13}C-enriched at carbon C24, have been estimated by Cabral et al.[63] from temperature-dependent NMR spectral changes. It was found that the deprotonated bile salts at pH 10 have half-times for flip-flop of greater than 24 h. The recent experiments of Donovan and Jackson,[63] however, are in contradiction to these earlier results. Donovan and Jackson showed a flip-flop for the charged bile salts in much shorter time, i.e., hours, and reported that the flip-flop rate increased with bile salt concentration in the membrane.[64]

In our ITC measurements for the determination of the partition coefficient where a lipid vesicle suspension was titrated with a monomeric anionic surfactants (SDS or bile salts) we found that the observed curves could not be simply simulated using the partitioning model where both monolayers were accessible to the surfactant. This indicated that the surfactant flip-flop was probably slow under these conditions where only small amounts of these molecules were incorporated into the outer monolayer. This is one reason we determined the partition coefficient from the reverse titration experiment, i.e., injecting a lipid vesicle dispersion into monomeric bile salt solution. In this case always a high concentration of surfactant is present, enough to make the bilayers permeable enough for complete equilibration to both monolayers. Also in the solubilization experiments the added surfactant solution had a much higher concentration and the expected concentration of the detergent in the outer monolayer was after the first injections already high enough to make the bilayers more permeable. Our ITC titration curves indicated half-times of maximally 4−5 min and not hours. The reason for the much faster flip-flop was the high temperature of 60 °C used for the experiments with DPPC. Assuming an activation energy of 80 kJ·mol^{-1}, as observed for other surfactants (Gutewort and Blume, unpublished data), one can expect a reduction of the half-times for flip-flop by a factor of ~30 going from 25 to 60 °C. Therefore, our results for fast flip-flop of charged bile salts once a sufficiently high amount of bile salt is bound to the bilayers is in agreement with the recently published results on flip-flop rates by Donovan and Jackson.[64]

Solubilization of DMPC in Water. Solubilization ITC experiments were also performed with the phospholipid DMPC to characterize the influence of the alkyl chain length on the incorporation of detergent and detergent induced solubilization of the membrane. For this purpose only NaDC/DMPC mixtures in water were investigated. The DMPC measurements were carried out at $T = 30$ °C, because in this case the main phase transition temperature T_m is 24.0 °C. Figure 9 shows a solubilization ITC curve of a 6 mM DMPC vesicle dispersion by a 150 mM NaDC solution in water, and Figure 10, the resulting phase diagram. The R_e^{sat} and R_e^{sol}, as well as the D_w^{sat} and D_w^{sol}, values are summarized in Table 2. The required detergent concentration for solubilization is significantly lower for DMPC with the shorter chains compared to DPPC. The difference between the D_w values is about 2 mM. Also, the slopes of the phase boundaries are smaller and almost identical ($R_e \sim 0.065$). This implies that the amount of detergent in the mixed vesicles and micelles is even lower for the PC with the C_{14} chains compared to C_{16} analogue.

As already mentioned above, PCs with saturated chains are more susceptible to solubilization than their counterparts with unsaturated chains and, in addition, bilayers with the shorter chain PCs are even more unstable. This

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(64) Donovan, J. M.; Jackson, A. A. *Biochemistry* **1997**, *36*, 11444.

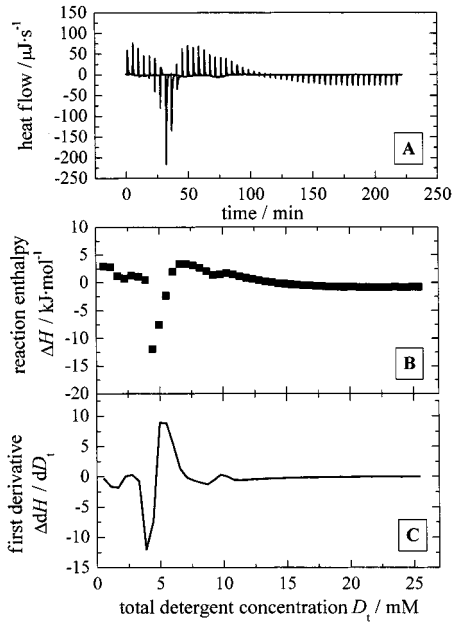

Figure 9. Solubilization experiment: Titration of a micellar NaDC solution (150 mM) into a DMPC dispersion (6 mM) in H_2O with $50 \times 5\,\mu$L injections at $T = 30$ °C. Key: (A) calorimetric traces; (B) normalized titration heat Q vs total detergent concentration in the cell D_t; (C) first derivative of curve B.

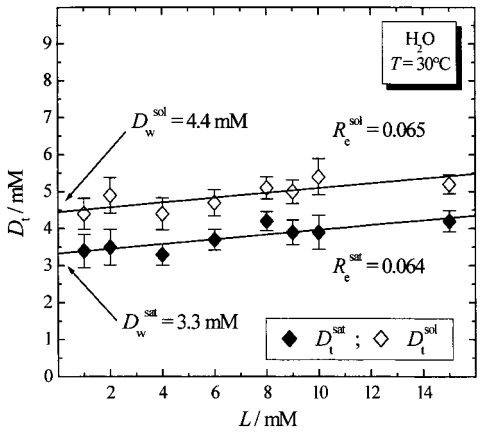

Figure 10. Phase diagram for the vesicle-to-micelle transition in NaDC/DMPC system in H_2O at $T = 30$ °C. Phase boundaries are shown with the D_t^{sat} (filled) and D_t^{sol} (open) values for DMPC concentrations between 1 and 15 mM. The R_e^{sat} and R_e^{sol} values were obtained from linear least-squares fits of the experimental data, with the D_w^{sat} and D_w^{sol} values from the extrapolation to $L = 0$ mM.

can also be explained by the fact that it is easier to form positively curved surfaces as they occur in micelles with saturated phospholipids, particularly when their chain length is short. Phospholipids with unsaturated chains have a larger tendency for negative spontaneous curvature. Therefore, unsaturated PCs are more stable toward transformation into micelles with their positively curved interface.[65]

DSC Studies of DPPC/Bile Salt Mixtures. The observed ITC solubilization curves of fluid lipid membranes imply that the bilayers become permeable already at low bile salt concentration in the bilayer. Saturated PCs form lamellar gel phases at lower temperature. The question arises whether the gel phase bilayers are also converted to micelles at the same saturation concentration

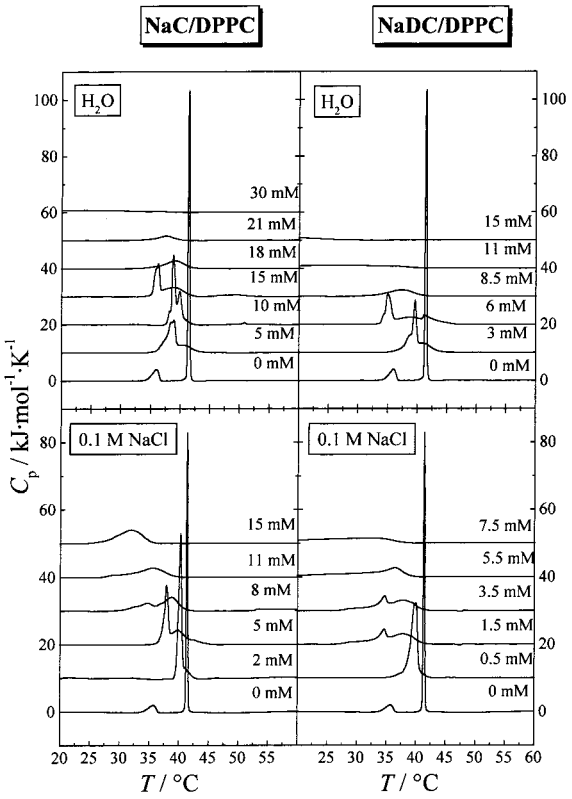

Figure 11. DSC heat capacity curves of DPPC in the presence of different amounts of NaDC and NaC in water and 0.1 M NaCl at pH 7.4.

or whether the bile salts are excluded from the gel phase. ITC experiments with gel phase vesicles are difficult to perform because of the slow kinetics of incorporation and flip-flop. However, the effects of bile salts on the gel phase can easily be studied by differential scanning calorimetry (DSC). Figure 11 summarizes the thermotropic behavior of DPPC in the presence of different amount of bile salts. For pure DPPC in water as well as in 0.1 M NaCl, the $L_{\beta'} \rightarrow P_{\beta'}$ phase transition (pretransition) is observed at ~36 °C, and the $P_{\beta'} \rightarrow L_\alpha$ phase transition (main transition), at 41.5 °C. The presence of small amounts of bile salts induces the abolishment of the pretransition and a decrease of the main phase transition temperature. With increasing amount of bile salt, two peaks were observed, a sharper phase transition at lower temperature followed by a second broader peak at higher temperature. Both phase transitions occurred at lower temperatures compared to the main phase transition temperature of pure DPPC (see Figure 11). The reason for the appearance of these two DSC peaks is a phase coexistence of gel phase and liquid-crystalline phase with different compositions over a larger temperature range. Similar results of DSC experiments in taurocholate/DPPC systems were reported by Forte et al.[66] With increasing bile salt concentration, the phase transition becomes broader and then vanishes completely as can be seen from Figure 11. However, the complete disappearance of the DSC peak only occurs at much higher concentrations than necessary for the complete transformation of liquid-crystalline vesicles into mixed micelles. This means that the gel phase bilayers contain less surfactant and that upon heating a transformation of gel phase mixed bilayers in coexistence with mixed micelles occur into completely mixed micelles. The

(65) Kozlov, M. M.; Lichtenberg, D.; Andelman, D. *J. Phys. Chem. B* **1997**, *101*, 6600.

(66) Forte, L.; Andrieux, K.; Keller, G.; Grabielle-Madelmont, C.; Lesieur, S.; Paternostre, M.; Ollivon, M.; Bourgaux, C.; Lesieur, P. *J. Therm. Anal.* **1998**, *51*, 773.

exact composition of these different types of aggregates can only be determined when the total phase diagram is known. This is not the case, and only very few lipid−surfactant systems exist with lipids with saturated chains where a complete temperature−composition diagram has been determined in detail.[67,68]

Summary and Conclusions

We have shown that ITC is a fast and convenient method for the determination of interaction parameters of surfactants with lipid vesicles. We performed experiments with the two bile salts cholate and deoxycholate and vesicles of the saturated phosphatidylcholines DPPC and DMPC. The results of the partition experiments showed that when bile salts are added to vesicles at low concentration, the incorporation first occurs into the outer monolayers of the vesicles and the flip-flop is too slow on the time scales of the experiments to obtain reliable partition curves. When the lipid vesicles are added to the bile salts, incorporation and flip-flop are fast enough to obtain reproducible results. The ITC curves could be simulated using a partitioning model where electrostatic effects are incorporated. The experiments show that the transfer of bile salt molecules from a micelle to a bilayer is endothermic at high temperature. The results from the partitioning experiments agree with those obtained from the solubilization experiments where lipid vesicles were titrated with concentrated bile salt solutions to obtain complete solubilization of the phospholipid into mixed micelles. The observed solubilization curves could be understood using the results from partitioning and demicellization experiments. The phase diagrams were different for systems with and without salt. In salt-free solutions, the vesicles become unstable at much lower effective detergent concentration than in 0.1 M NaCl solution. In addition, the phase boundaries are curved at low lipid concentration indicating significant contributions to the free energy from end-caps of cylindrical micelles. Comparison with other surfactants shows that bile salts are much more effective in solubilizing phosphatidylcholines than surfactants with alkyl chains and that bilayers of saturated lipids are more unstable than those of unsaturated lipids due to the larger tendency of saturated lipids for surfaces with positive curvature.

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Appendix

We used in our calculation a partition coefficient P in mole fraction units, i.e., defined as the ratio of the detergent mole fraction bound in the aggregates x_b and free in water x_w:

$$P = \frac{x_b}{x_w} \tag{A1}$$

Both mole fractions can be described in terms of the concentration of lipid L, detergent bound in the aggregates D_b, and free in water D_w as well as the water concentration W (=55.5 M):

$$x_b = \frac{D_b}{D_b + L} \tag{A2}$$

$$x_w = \frac{D_w}{D_w + W} \tag{A3}$$

The adsorption and incorporation of bile salt molecules generate a negatively charged membrane surface, which has to be taken into account. A correction due to the electrostatic effects can be included once the surface charge density is known.

The calculation of the surface charge density σ of the bilayer was performed as described by Kuchinka and Seelig:[53]

$$\sigma = ze_0 \frac{\frac{D_b}{LA_L}}{1 + \frac{D_b A_D}{LA_L}} \tag{A4}$$

z is the electric charge of the detergent, e_0 is the elementary electric charge, A_L is the average surface area of a DPPC molecule ($A_L \approx 65$ Å^2),[54] and A_D the average surface area of a bile salt molecule ($A_D \approx 40$ Å^2).[40]

The surface potential ψ_0 can then be calculated using the Grahame equation for 1:1 electrolytes:[55]

$$\psi_0 = -k_B \frac{T}{e_0} a \cosh\left(\frac{\sigma^2}{4\epsilon_0\epsilon_r RT(1000c_{el})} + 1\right) \tag{A5}$$

Here k_B is the Boltzmann constant, T the absolute temperature, e_0 the elementary electric charge, ϵ_0 the permittivity of free space, ϵ_r the dielectric constant of water, R the gas constant, and c_{el} the electrolyte concentration. Using the electrostatic correction and the fact that the detergent shows nonideal mixing behavior with the lipid in the membrane,[35] one obtains for the apparent partition coefficient P

$$P = P_0 \exp\left(-\frac{ze_0\psi_0}{k_B T}\right) \exp\left(-\rho\frac{(1 - x_b)^2}{RT}\right) \tag{A6}$$

P_0 is the intrinsic partition coefficient for detergents in the presence of lipid vesicles. The first exponential takes into account the correction due to electrostatics, and the second exponential is the correction due to nonideal mixing with ρ being the nonideality parameter.

The concentration of bound detergent D_b is expressed as a function of D_t and lipid concentration L by

$$D_b = \frac{1}{2}(D_t - L) - \frac{W}{2P} + \sqrt{\frac{1}{4}(D_t + L)^2 - \frac{1}{2}(D_t - L)\frac{W}{P} + \frac{1}{4}\left(\frac{W}{P}\right)^2} \tag{A7}$$

From the total and bound detergent concentrations D_t and D_b the bulk concentration of the detergent D_{wbulk} can be expressed by

$$D_{wbulk} = D_t - D_b \tag{A8}$$

Taking the Gouy−Chapman[56] theory into account, the detergent concentration at the membrane surface D_w can

(67) Madler, B., Klose, G.; Mops, A.; Richter, W.; Tschierske, C. *Chem. Phys. Lipids* **1994**, *71*, 1

(68) Madler, B.; Binder, H.; Klose, G. J. *Colloid Interface Sci.* **1998**, *202*, 124.

be calculated as

$$D_w = D_{wbulk} \exp\left(-\frac{ze_0\psi_0}{k_B T}\right) \quad \text{(A9)}$$

The change of the concentration of bound detergent in the vesicles as a function of the injected phospholipid dispersion is obtained by the derivative which is calculated numerically from the concentrations D_b and L after the nth and $(n + 1)$th addition of vesicles using eq A7:

$$\frac{\Delta D_b}{\Delta L} = \frac{D_{b(n+1)} - D_{b(n)}}{L_{n+1} - L_n} \quad \text{(A10)}$$

The normalized titration heat ΔH is composed mainly of two effects: (1) the transfer enthalpy ΔH^T of the incorporation of bile salt monomers into the lipid bilayers; (2) the heat of dilution ΔH_{dil} of the phospholipid dispersion. The normalized titration heat ΔH can be described by

$$\Delta H = \frac{\Delta D_b}{\Delta L} \Delta H^T + \Delta H_{dil} \quad \text{(A11)}$$

The ΔH_{dil} values are determined by titrating the phospholipid dispersion into pure solvent. The experimentally observed reaction heats Q are related to the normalized heat of titration ΔH by multiplication with the total lipid concentration in the syringe $L_{t,syr}$ and the injection volume v_{inj}:

$$Q = \Delta H L_{t,syr} v_{inj} \quad \text{(A12)}$$

The partition coefficient P_0 and the transfer enthalpy ΔH^T are obtained by nonlinear least-squares fit of the experimental ΔQ vs L diagrams using eqs A7−A12. The calculated curves are shown in Figure 3B′,B″ as solid lines.

For the calculation of the changes in Gibbs free energy $\Delta G^T_{\#}$ the values for the intrinsic partition coefficient P_0 and the average partition coefficients P_{av} for the whole concentration range ($P_{\#}$; index "#" means "0" or "av") were used according to

$$\Delta G^T_{\#} = -RT \ln P_{\#} \quad \text{(A13)}$$

The entropy change $\Delta S^T_{\#}$ was then calculated from the values of the transfer enthalpy (eq 11) and the Gibbs free energy (eq A13) using the Gibbs−Helmholtz equation:

$$T\Delta S^T_{\#} = \Delta H^T - \Delta G^T_{\#} \quad \text{(A14)}$$

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