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Stability of liposomal formulations: action of amphiphilic molecules

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Abstract

The destabilization of vesicles obtained by cyclic freezing-thawing and extrusion (FATVET) bilayer membranes consisting of L-α-dipalmitoyl phosphatidylcholine (DPPC) by the bile salt sodium deoxycholate (DOC) was studied from turbidimetric, entrapped solute release and steady-state fluorescence anisotropy measurements. Based on turbidity measurements, bilayer membranes in a rigid gel state are more resistant to lysis by deoxycholate than are bilayers in a fluid liquid-crystal state. Thus, the $[\text{detergent}]_{\text{total}}/[\text{phospholipid}]$ mole ratio, R_t^{sat}, needed to saturate the bilayers at 25°C ($T < T_m$) is three times greater than that at 50°C ($T > T_m$) also, R_t^{sol}, which corresponds to complete solubilization of the bilayers, is twice higher at 25°C than it is at 50°C. However, this does not mean that bilayer saturation or complete solubilization calls for increased DOC concentrations, as shown by the fact that the effective mole ratios for both events, R_e^{sat} and R_e^{sol}, are identical at 25°C and 50°C. The ability of DOC molecules to intercalate themselves between DPPC molecules therefore appears to be decreased by tightly packed phospholipid molecules. Thus, the partition coefficient for the sub-solubilization range, R_e, is almost 3 times lower at 25°C than it is at 50°C. On the other hand, the R_e^{sat}, R_e^{sol} and K values for FATVETs{egg yolk lecithin (EYL)} at 25°C are virtually identical with those for FATVETs{DPPC} at 50°C. Hence, for a series of phospholipid analogues, the partition coefficient K for a surfactant/liposome system is bound to depend primarily on the physical state of the bilayer. The retention properties of FATVETs{DPPC} are impaired at sub-lytic DOC levels thus, 5-fluorouracil (5-FU) leakage under these conditions amounts to $R_e^{50\%} = 0.09$, which is somewhat smaller than R_e^{sat} (1.2–1.4). This may be the result of the packing and structural changes undergone by lamellae prior to solubilization of the bilayers.

Abbreviations: EYL, egg yolk lecithin; DPPC, L-α-dipalmitoyl phosphatidylcholine; DOC, sodium deoxycholate; 5-FU, 5-fluorouracil; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tri(hydroxymethyl)amino methane; MLVs, multilamellar vesicles; FATVETs, vesicles obtained by cyclic freezing-thawing and extrusion; V_i, overall internal volume of the liposomal suspension; PCS, photon correlation spectroscopy; D_t, total detergent concentration in the medium; D_w, detergent concentration in the aqueous medium; D_b, detergent concentration in bilayer; R_e, [detergent]/[lipid] effective mole ratio; R_e^{sol}, R_e at complete solubilization; R_e^{sat}, R_e at the onset of the lamellar-to-micelle transition; $R_e^{50\%}$, [detergent]/[lipid] effective mole ratio that induces the release of 50% of the liposomal content; S.D., standard deviation.

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The 1,6-diphenyl-1,3,5-hexatriene (DPH) steady-state fluorescence anisotropy results also suggest that DOC alters acyl chain packing, diminishes transition cooperativeness and decreases the orderliness of acyl chains. © 1997 Elsevier Science B.V.

Keywords: Deoxycholate; Surfactant-liposome interactions; Drug delivery systems; 5-fluorouracil; Extrusion; Steady-state fluorescence anisotropy; DPH

1. Introduction

Like some soluble amphiphilic components of biological fluids, surfactants induce morphological and permeability changes in cell membranes that alter their functionality. The effects of surfactants (detergents) on liposomal structures have been the subject of much study in various respects (Lasch, 1995). Surfactants are widely used for membrane protein solubilization and reconstitution in laboratory applications, as well as for producing controlled-size liposomes.

Liposome-detergent interactions play a central role in liposome-mediated drug delivery mechanisms. Ideally, liposomes, like any delivery system, must transfer the drug directly to the target organ and keep it there for as long as required; some amphiphilic molecules in physiological fluids can release some drug from liposomes, however. Bile acids and salts are amphiphilic physiological substances and they have been widely used in studies on the detergent-membrane interaction. While they interact with liposomes in a somehow different way from non-ionic and ionic surfactants, the mechanisms by which lipid bilayers are solubilized are similar for all (Lasch and Schubert, 1993). The action of bile derivatives on liposomes has been studied by using a variety of techniques. In most cases, the results were consistent with the 'thread-like' model for a mixed micelle structure (Walter et al., 1991).

The purpose of this work was to investigate the destabilizing effects of deoxycholate on bilayers of phosphatidylcholine liposomes used as drug delivery systems. 5-FU encapsulation and release by liposomes were previously studied elsewhere (Elorza et al., 1993). We used deoxy cholate as a model for small, biologically occurring amphiphilic molecules. Because packing of acyl chains in liposomal bilayer is changed by amphiphiles, we also investigated the effect of the surfactant on the drug retention ability of the lipid vesicles. Finally, in order to shed some light on the mechanism of action of deoxycholate, we studied its effects on lipid organization through the variation of steady-state fluorescence anisotropy measurements of DPH as a function of temperature.

2. Materials and methods

2.1. Lipids and other reagents

Lyophilized egg yolk lecithin (EYL, $>98\%$ pure), L-α-dipalmitoyl phosphatidylcholine (DPPC, 99% pure), sodium deoxycholate (DOC), 5-fluorouracil (5-FU) and the fluorescent probe 1,6-diphenyl-1.3.5-hexatriene (DPH) were supplied by Sigma. Tritiated 5-fluorouracil ([^{3}H]5-FU) was obtained from Amersham as an aqueous solution of 5 mCi (185 mBq) activity.

The stated purity of the phospholipids was checked by the usual thin layer chromatographic methods (Kovács et al., 1986). They were stored as 2:1 v/v chloroform/methanol solutions in a nitrogen atmosphere at -20°C. Phospholipids were titrated according to Bartlett (Bartlett, 1959). Deoxycholate was recrystallized twice from hot ethanol. DPH was stored as a 1 M tetrahydro furan (THF) solution in the dark. [^{3}H]5-FU was stored as an isotopic dilution of 5 μCi ml^{-1} specific activity in isotonic tri(hydroxymethyl)amino methane (Tris) buffer. The polycarbonate filters used were purchased from Nucleopore. All other reagents were analytical-grade chemicals and bidistilled water was used throughout.

2.2. Preparation and characterization of vesicle dispersions

Multillamelar vesicles (MLVs) were formed from dried lipid films by resuspension in an aqueous buffer containing 5 mM Tris–HCl and 150 mM NaCl (pH 7.4, 290 mOsm), following rehydration in a nitrogen atmosphere for 2 h. The initial phospholipid concentration in the aqueous medium was 60 μmol ml^{-1} for all preparations. [^{3}H]5-FU was added at a 2 μCi ml^{-1} activity level to the buffer solution as required. The osmolality of the solutions was measured with a Knauer digital cryo-osmometer. Freeze-thaw extrusion vesicles (FATVETs) were prepared as described (Chantres et al., 1996). Extrusion was performed at 25°C for FATVETs{EYL} and 55°C for FATVETs{DPPC}.

Vesicle dispersions were characterized from their internal volume, V_i, average vesicle size and size distribution and lamellarity as described elsewhere (Chantres et al., 1996). Vesicles shown an average diameter of 98 ± 7 nm (polydispersity index 0.05) and were predominantly unilamellar, as estimated by ^{31}P-NMR. The average V_i value $\pm$ S.D. (expressed in litres of encapsulated water per mole of phospholipid) were 1.90 ± 0.08 l mol^{-1}.

2.3. Turbidity measurements

The solubilization of phospholipid vesicles by DOC was monitored turbidimetrically. A volume of 2.0 ml of vesicle suspension was placed in a cuvette and supplied with a preset volume (10–50 μl) of DOC stock solution stepwise. The initial phospholipid concentration in the samples was 3.0 mM. The final concentrations of the lipid and surfactant were calculated from their initial values and the volume of surfactant solution added. On mixing, samples were maintained at the experimental temperature for exactly 30 min prior to measurement.

The absorbance (turbidity) of each vesicle suspension was measured against a blank as a function of the overall surfactant concentration using a Beckmann DU7 spectrophotometer at 500 nm. During measurements, turbidity was checked to remain at steady-state level for 5 min, the cuvette being shaken at 30 s intervals. The temperature of the vesicle suspensions was controlled by means of a Peltier system and a probe immersed in the cuvette. Results are given as percentages of the initial turbidity of the samples containing no surfactant.

2.4. Determination of 5-FU release

The amount of [^{3}H]5-FU released from FATVETs{DPPC} at 25°C was taken to be a measure of structural integrity of the liposomes. As noted earlier, lipid films were hydrated in a buffer containing [^{3}H]5-FU at a 2 μCi ml^{-1} activity. Excess, non-encapsulated drug was removed from the liposomal preparation by passing it through a chilled 1.5×7.0 cm Sephadex G75 column. Release experiments were started by supplying a volume of 1.5 ml of FATVETs{DPPC} dispersion with appropriate volumes of DOC stock solution and isotonic buffer to a final volume of 2.0 ml (the mixture was thus made 2.8 mM in phospholipid). Next, 0.5 ml aliquots of the liposomal suspension were placed in pre-washed collodion bags (Sartorius, M_r cut-off 20 kDa). The bags were transferred into test tubes of 2.5 cm i.d. containing 10 ml of buffer and the tubes stoppered and immersed in a thermostated bath at 25°C. In order to ensure homogeneity in the dialysis medium, the tubes were stirred magnetically throughout the incubation period. The amount of drug released was monitored at 120 min by counting dialysate aliquots diluted with Normascin 22 cocktail on an Analytical Kontron BETAmatic scintillation counter. The amounts of 5-FU released, corrected for passive flux (i.e. in the absence of DOC), are given as percentages of those initially entrapped by the vesicles. Test controls revealed that the combined amount of label released at the end of each experiment and that remaining inside the vesicles (as determined after lysis with Triton X-100), coincided to within 5% with the amount initially entrapped by the vesicles. Also, preliminary control experiments revealed that 5-FU dialysed freely across the dialysis membranes used and that significant adsorption of the cytostatic agent by the membranes

occurred. In the experiments, 0.5 ml aliquots of buffer containing [^{3}H]5-FU at a 0.1 μCi ml^{-1} activity were placed in dialysis bags and the bags transferred into test tubes containing 10 ml of buffer, the whole being stirred magnetically. At appropriate times (2, 5, 10, 30 and 60 min), aliquots from the bags and the dialysate were counted as described above. The results showed both solutions to contain the same concentration of 5-FU ($\pm$1%) from 2 min on. Next, the bags were thoroughly rinsed with non-tritiated 5-FU solution and aliquots from the rinsing liquid were checked for [^{3}H] activity, which was never observed.

Phospholipid solubilization by deoxycholate was also checked in each sample: surfactant-treated FATVETs{DPPC} vesicles were ultrafiltered through Centrisart devices (Sartorius, M_r cut-off 20 kDa) and lipid phosphorus in the filtrates assayed according to Bartlett (Bartlett, 1959). The results are given as percent solubilization values relative to the initial amount of phospholipid in the samples.

2.5. Fluorescence anisotropy measurements

The steady-state anisotropy of DPH embedded in lipid bilayers were monitored as described elsewhere (Chantres et al., 1996). Steady-state anisotropy values were calculated from the following equation:

$$r_s = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the parallel and normal component, respectively, of the fluorescence emission relative to the vertical polarization plane of the excitation beam and G is the Azumi-McGlyn factor (Azumi and McGlynn, 1962).

The r_s values thus obtained were used to construct Arrhenius plots in order to determine the effect of temperature on bilayer 'microviscosity', $\bar{\eta}$, as measured from DPH motion. We used the relation of van der Meer et al. (van der Meer et al., 1986) to estimate it:

$$6D\tau = \frac{r_0}{r_s} - 1 + m \quad (2)$$

where D is the wobbling diffusion constant for rod-like probes such as DPH, τ the fluorescence lifetime, r_0 the maximum fluorescence anisotropy and m, a positive quantity, the difference between the rotational diffusion of the probe in the membrane and that in a reference isotropic oil. In this work, we assumed $m = 1.7$ and $r_0 = 0.4$ (van der Meer et al., 1986). We used Arrhenius plots to derive the enthalpy of activation per flow unit, $\Delta H^{\neq}$. For a lipid system that undergoes no phase separation or transition, $\Delta H^{\neq}$ is assumed to be temperature-independent; hence, a plot of log $\bar{\eta}$ versus $1/T$ should be a straight line. The flow activation energy can be interpreted as the energy required to dissociate a molecule (or mole) from the bulk mass, so it must increase with increasing number of degrees of freedom for interactions between molecules. Consequently, $\Delta H^{\neq}$ can be used as a measure of orderliness in the hydrocarbon core of lipid bilayers.

3. Results and discussion

The critical micellization concentration (CMC) of deoxycholate in the aqueous medium used were 2.7 mM, as determined by using the fluorescent dye method (Chantres et al., 1996).

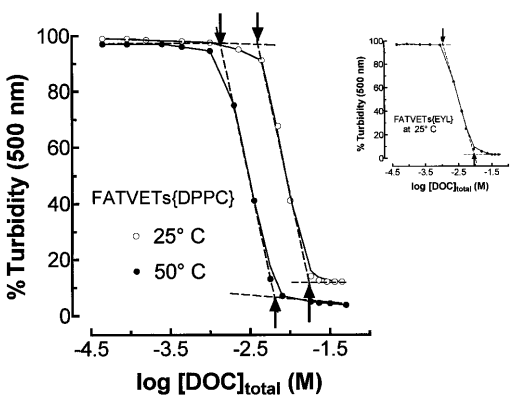

Fig. 1. Changes in the turbidity of FATVETs{DPPC} dispersions at 500 nm as function of deoxycholate concentration in the medium (○) at 25°C (bilayer in the gel state), and (●) at 50°C (bilayer in the liquid crystal state). Inset: data for FATVETs{EYL} dispersions at 25°C. Arrows pointing downwards and upwards correspond to bilayer saturation and complete bilayer solubilization, respectively.

Fig. 1 shows typical turbidimetric results obtained for FATVETs{DPPC} at 25 and 50°C. For comparison, the results obtained for FATVETs{EYL} at 25°C are included in the figure inset. Turbidity changes in the vesicle suspensions arising from the stepwise addition of DOC have been rationalized in terms of the three-stage model (Schubert et al., 1986; Lichtenberg et al., 1983; Helenius and Simons, 1975). Initially, the surfactant partitions between the bilayer and the water phase (stage I). As the surfactant concentration is raised, bilayer saturation is gradually reached (arrowheads pointing downwards in Fig. 1). On saturation, the bilayer is 'solubilized'; in the process, bilayers coexist with mixed micelles (stage II) and a turbidity decay is observed. Finally, a critical lipid/surfactant ratio is attained (arrowheads pointing upwards in Fig. 1) where all bilayers have been replaced by mixed micelles of a small particle size. These events were previously studied by ^{31}P-NMR spectroscopy and electron microscopy, which provided results similar to those derived from turbidity measurements (Paternostre et al., 1988).

Correct rationalization of the surfactant effects on vesicles requires an exact knowledge of the 'effective' detergent/lipid mole ratio, R_e (i.e. that in the bilayer), which may be significantly different from the 'total' mole ratio (referred to the total concentration of surfactant in the medium).

At sub-solubilizing detergent concentrations, a partition equilibrium between the bilayer and aqueous medium is assumed to exist (Schubert et al., 1986). Hence, the partition coefficient can be defined as

$$K = \frac{D_b/PL}{D_w} \quad (3)$$

where D_w and D_b are the equilibrium detergent concentration in the aqueous phase and the bilayer, respectively and PL the total concentration of phospholipid. Since, $R_e = D_b/PL$, Eq. (3) can be rewritten as

$$K = \frac{R_e}{D_w} \quad (4)$$

On the other hand, since the total detergent concentration is $D_t = D_w + D_b$, one can write

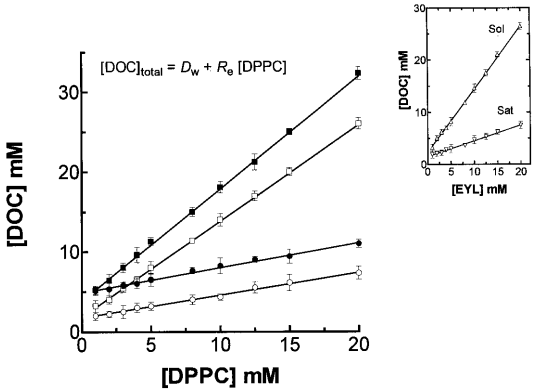

Fig. 2. Estimation of R_e and D_w. Variation of the DOC concentration corresponding to the onset (□) and completion (○) of lipid bilayer solubilization, as a function of the phospholipid concentration. Empty symbols correspond to 25°C and filled symbols to 50°C. Inset: results obtained at 25°C in FATVETs{EYL}. Bars symbolize S.D. values obtained from six individual experiments.

$$D_t = D_w + R_e PL \quad (5)$$

This linear relation between D_t and PL allows the ready estimation of R_e^{sat} and R_e^{sol} (viz. the detergent/phospholipid effective mole ratio needed to saturate phospholipid membranes and that in the mixed micelles, respectively) for any liposome sample. In addition, K can also be estimated from the ratios of the slopes (R_e^{sat}) to the intercepts at a zero lipid concentration (D_w).

Fig. 2 shows the variation of the DOC concentrations corresponding to the onset of solubilization and total solubilization with the total phospholipid concentration at 25 and 50°C. The curves obtained at both temperatures are strikingly linear ($r^2 \geq 0.993$). The inset of Fig. 2 depicts the results obtained for FATVETs{EYL} at 25°C. Table 1 shows the R_e^{sat}, R_e^{sol}, D_w (aqueous detergent concentration in equilibrium with saturated bilayers and saturated micelles) and K values obtained. As can be seen, for FATVETs{DPPC}, the respective effective mole ratio (R_e^{sat} and R_e^{sol}) were identical at the two temperatures. The detergent concentrations in the aqueous medium (D_w), in equilibrium with saturated bilayers or mixed micelles, were higher at 25 than at 50°C. The partition coefficient, K, which describes the detergent partitioning between the

lipid and aqueous phase, was almost three times higher at 50°C than it was at 25°C. Therefore, the ability of DOC molecules to penetrate through hydrocarbon chains is reduced by tight structural packing relative to a fluid state. In macroscopic terms, one may state that the surfactants partitions between water and the lipid phase in a gel state as poorly as it does in a liquid crystal state. On the other hand, it should be noted that the R_e^{sat}, R_e^{sol} and K values for FATVETs{EYL} at 25°C were virtually identical with those for FATVETs{DPPC} at 50°C. Under these conditions, the bilayers of both systems occur in the liquid crystal state. The amount of surfactant that the bilayers can accept depends on the packing density of the phospholipid molecules, as well as on surfactant-phospholipid interactions within the bilayers. Thus, for a series of phospholipid analogues, the partition coefficient K for a surfactant-liposome system is bound to depend primarily on the physical state of the bilayer. If such is the case, the temperature-dependence of K will jump abruptly over the transition temperature. Research aimed at confirming this hypothesis is currently under way.

Table 1
Solubilization parameters for EYL and DPPC vesicles in the presence of deoxycholate and estimated partition coefficients, below ($T = 25$°C) and above ($T = 50$°C) the main phase transition temperature of DPPC bilayers

	R_e (mol/mol)	D_w (mM)	K (mM^{-1})
Temperature 25°C			
FATVETs{EYL}			
Onset of solubilization	0.3	1.5	0.20
Complete solubilization	1.3	2.2	—
FATVETs{DPPC}			
Onset of solubilization	0.3	4.9	0.06
Complete solubilization	1.4	3.8	—
Temperature 50°C			
FATVETs{DPPC}			
Onset of solubilization	0.3	1.7	0.16
Complete solubilization	1.2	1.8	—

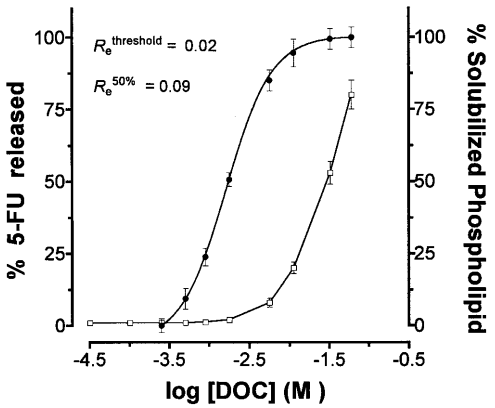

Fig. 3. (●) Percentage of 5-FU released and (□) percentage of phospholipid solubilized from FATVETs{DPPC} as a function of DOC concentration after 120 min of deoxycholate addition, at 25°C. The passive flux of drug has been subtracted. Bars symbolize S.D. values obtained from six individual experiments.

The action of surfactants on bilayers can also be assessed by examining their effects on the release of liposomal contents (Goñi et al., 1993). Several studies involving 5(6)-carboxy fluorescein as an entrapped label have been aimed in this direction. Our group monitored the release of 5-FU, a cytostatic drug and a firm candidate for liposomal encapsulation as a vehicle for delivery. Fig. 3 shows the percentage of 5-FU release from a FATVETs{DPPC} suspension at 25°C after 120 min. 5-FU is a small molecule ($M_r = 131$ Da) sparsely soluble in water, with an octanol/buffer partition coefficient of 0.15–0.20 at 20°C at pH 7.4, where it occurs in neutral form (pK_a 8–13). Passive leakage across DPPC bilayers under our experimental conditions was subtracted from all measurements. The leakage pattern observed was very similar to those reported by others for the cholate-, n-octyl-β-D-glucopyranoside- and Triton X-100-induced release of liposomal contents. The release profile in Fig. 3 exhibits a threshold deoxycholate/lipid mole ratio corresponding to an overall DOC concentration of ≈ 0.3 mM; beyond that threshold, the profile acquires a sigmoidal shape.Eq. (4) and Eq. (5) allow R_e values in the

sub-solubilizing DOC concentration range to be estimated from

$$R_e = \frac{D_t}{\frac{1}{K} + PL} \quad (6)$$

Thus, the DOC/DPPC effective mole ratio corresponding to the threshold for 5-FU release is $R_e^{thr} = 0.02$ and the effective mole ratio causing 50% of the liposomal content to be released under our experimental conditions is $R_e^{50\%} = 0.09$; both are therefore smaller than R_e^{sat} (see Table 1), where bilayer solubilization starts at 25°C. These results are consistent with those obtained from phospholipid solubilization measurements. As can be seen in Fig. 1, solubilization occurs at total DOC concentrations well above those that induce 5-FU release.

One other interesting observation that relates turbidimetric and release results is the fact that, as well no turbidimetric changes occurred after 5 min of exposure to the surfactant as only a small fraction of encapsulated 5-FU was released after as long as 120 min. Our results are consistent with others previously reported. Mechanistic explanations for these results have so far relied mainly on the model of Schubert et al. (Schubert et al., 1986b), which assumes the formation of transient holes at stage I of the 'bilayer' solubilization process.

We used the fluorescence anisotropy results for DPH to check for DOC-induced alterations of the lipid bilayer matrix. Fig. 4 shows the variation of the steady-state anisotropy of DPH fluorescence as a function of temperature for FATVETs{DPPC} in the absence (Fig. 4a) and presence (Fig. 4b–f) of DOC at total concentrations in the range 0.4–10.0 mM, equivalent to DOC/phospholipid effective mole ratios at 25°C of 0.02–0.6. As can be seen, the thermotropic gel↔liquid crystal phase transition of DPPC molecules in the bilayer structure still took place at a DOC/phospholipid effective mole ratio of 0.12, as calculated from the r_s versus T profile (Fig. 4e). The transition vanished by a DOC/phospholipid effective mole ratio of 0.6 (Fig. 4f), beyond which the r_s versus T profile departed from the typical shape for a lamellar structure (a smooth decay in r_s); in fact, no trace of the transition remained above a DOC/phospholipid effective mole ratio of 0.3 (results not shown). It is interesting to note that, as established above from turbidity measurements, R_e^{sat} (onset of solubilization) was also 0.3 at 25 and 50°C; moreover, from our release results it follows that leakage was complete at a detergent/phospholipid effective mole ratio of ≈ 0.9. Therefore, our anisotropy, turbidity and release values are consistent and suggest that disappearance of the bilayer structure starts at a DOC/phospholipid effective mole ratio of $\approx 1{:}3$ and is completed at $R_e \simeq 1$. At this point, the liposomal suspension probably becomes a dispersion of mixed lipid-detergent micelles.

The 'microviscosity' concept has traditionally been misapplied to bilayers—viscosity is a macroscopic concept hardly applicable to a microenvironment as a lipid bilayer— however, it may still be used though with a different meaning (van der Meer et al., 1986). In fact, a comparison of the fluorescence anisotropy arising from depolarization motion in a rod-like fluorescent probe such as DPH embedded in an anisotropic medium (e.g. a bilayer) with the emission anisotropy of the same probe dissolved in an isotropic medium such as a reference oil allows one to derive a constant m for use in a modified version of the Perrin equation in order to estimate 'microviscosity' values or construct Arrhenius plots from steady-state fluorescence anisotropy measurements. Since m represents the difference between the rotational diffusion of the probe in the bilayer and that in the isotropic reference oil, if the emission has the same r_s value, then the fluorophore must rotate faster in the medium that restricts its movements than in an unrestrictive medium.

Fig. 5 shows the Arrhenius plots for the r_s versus T curves of Fig. 4a, c and e. The enthalpy of activation, $\Delta H^{\neq}$, was calculated by linear regression of the curves. This parameter is a measure of orderliness in the bilayer lipid matrix. If the bilayer undergoes no phase separation or transition on heating, then $\Delta H^{\neq}$ will be a constant; otherwise, if a transition occurs, then $\Delta H^{\neq}$ will be almost constant below and above the transition limits and increase sharply between them. Fig. 5a, obtained for FATVETs{DPPC} in

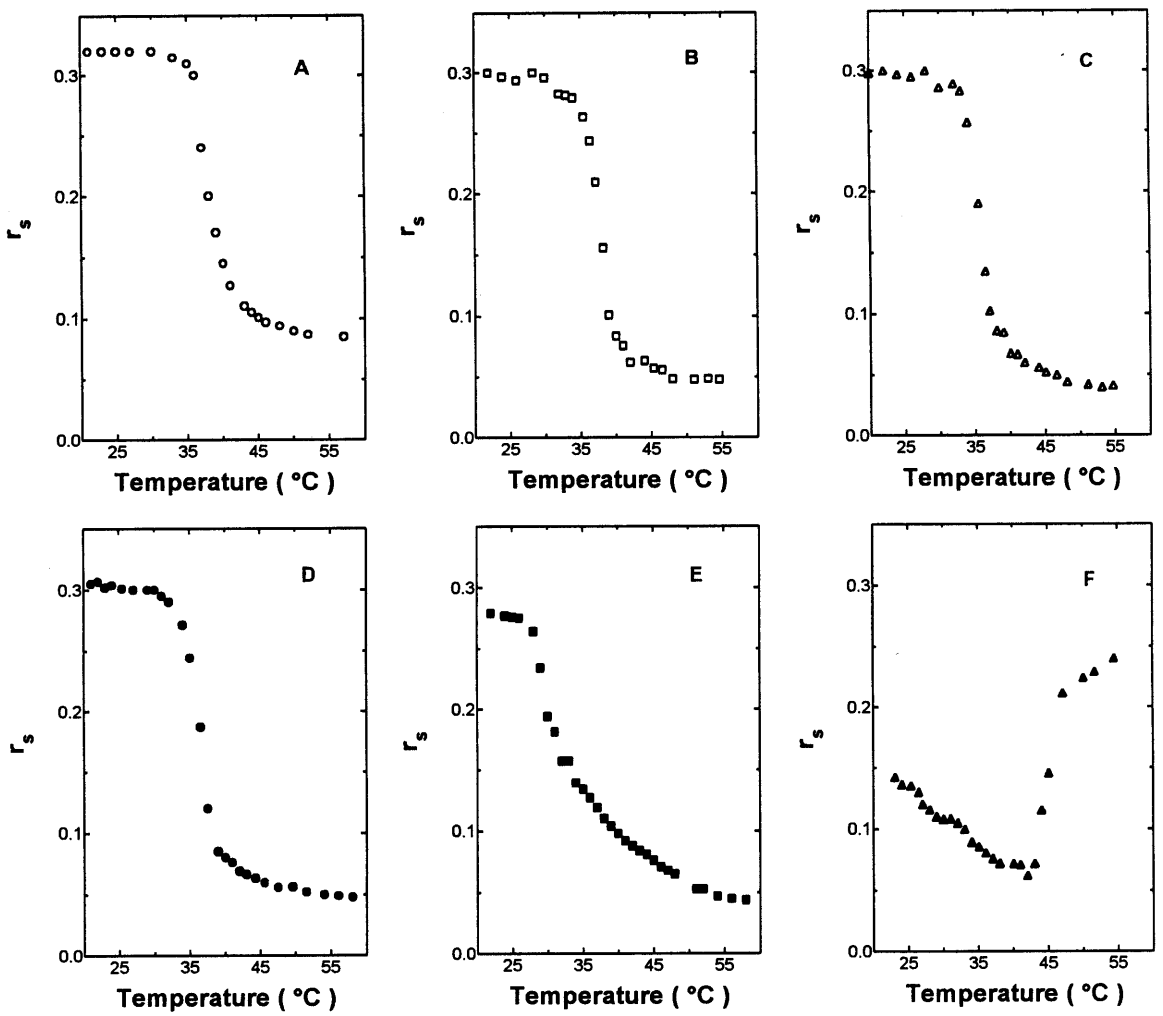

Fig. 4. Variation in the steady-state fluorescence anisotropy profiles (DPH vs. temperature in FATVETs{DPPC}) as a function of the total concentration of DOC in the medium. (a) without DOC; (b) 0.4 mM; (c) 0.8 mM; (d) 1.0 mM; (e) 2.0 mM; and (f) 10.0 mM.

the absence of detergent, is consistent with this description. Adding DOC at a detergent/lipid effective mole ratio up to 0.04 (i.e. up to about 0.8 mM DOC), decreased $\Delta H^{\neq}$ by about 30% without altering the overall appearance of the $\ln \bar{\eta}$ versus $1/T$ curves (Fig. 5b). Above $R_e = 0.06$ ($\approx$ 1.0 mM DOC), no unique linear least-squares fitting was possible in the transition range, so we adopted a double linear least-squares fitting as an acceptable solution (Fig. 5c). This behaviour probably reflects the occurrence of various bilayer domains (e.g. mixed lipid-DOC aggregates and lipid-rich domains) or distinct structures of mixed composition with disparate DPH motion characteristics. Above $R_e^{25°C} = 0.3$ (≈ 5.0 mM DOC), the bilayer was seemingly dissolved (results not shown), so estimating $\Delta H^{\neq}$ was pointless.

Highly sensitive DSC experiments (Spink et al., 1982; 1991), about the thermal behaviour of mixtures of DPPC and either taurocholate or taurodeoxycholate, revealed complex thermal transitions that changed dramatically with R_e, the

effect being greater for the dihydroxy bile salt. Absorbance and flow calorimetry experiments (Malloy and Binford, 1990) on mixtures of dimiristoylphosphatidylcholine (DMPC) or dilauroylphosphatidylcholine (DLPC) with cholate, deoxycholate and lithocolate also revealed the occurrence of several states between R_e^{sat} and R_e^{sol}.

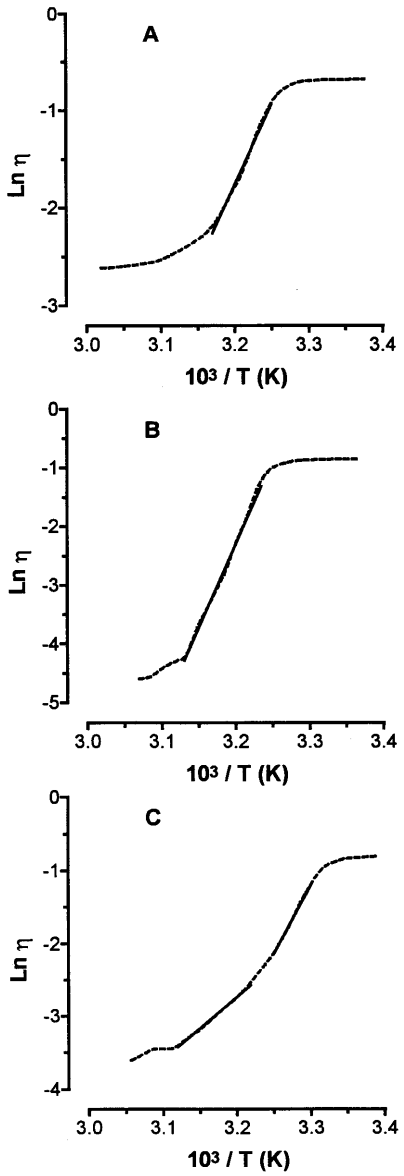

Fig. 5. Arrhenius plots of 'microviscosity' dependence on temperature in FATVETs{DPPC} bilayers as a function of the total concentration of DOC in the medium. (a) without DOC; (b) 0.8 mM; and (c) 2.0 mM. The straight line is the best linear regression fit within the temperature range and allows calculation of the activation energy for the process.

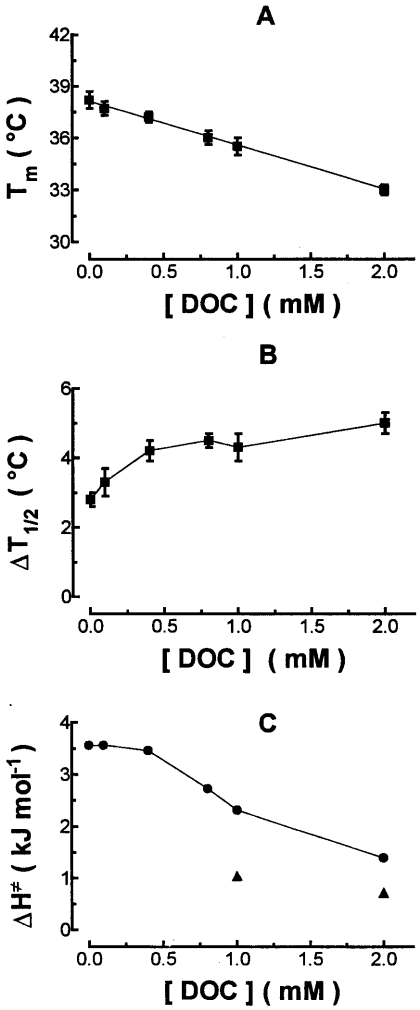

Fig. 6. Data estimated from steady-state fluorescence anisotropy results for FATVETs{DPPC} plotted against the DOC concentration: (a) Variation of the main transition temperature, T_m; (b) Variation of the transition width at half-height, $\Delta T_{1/2}$; (c) Variation of the activation enthalpy $\Delta H^{\neq}$.

The fluorescence results obtained are summarized in Fig. 6 as the variation of the main transition temperature (T_m), $\Delta T_{1/2}$ and the enthalpy of activation ($\Delta H^{\neq}$) with the DOC concentration. T_m and $\Delta T_{1/2}$ were estimated by polynomial least-squares fitting of r_s-T curves and subsequent derivation; T_m corresponded to the maximum and

$\Delta T_{1/2}$ to the width at half-height. In the absence of detergent, T_m was 38°C for FATVETs{DPPC}, as opposed to 41°C for DPPC bilayers in MLV structures (a result of their smaller radius). Addition of DOC caused T_m to decrease linearly throughout the concentration range studied. Based on the theory of regular solutions, the decay in T_m indicates that DOC is more 'soluble' in the liquid crystal phase than it is in the gel phase. Transition cooperativeness, as judged from $\Delta T_{1/2}$, also decreased with increasing DOC concentration in a virtually linear manner. Similarly, as judged from the enthalpy of activation, the orderliness of hydrocarbon chains also diminished from the beginning.

4. Conclusions

The effects of DOC on phosphatidylcholine bilayers can be summarized as follows:

(a) In terms of structural stability as judged from turbidity measurements, liposomal bilayer membranes in a rigid gel state—as in DPPC liposomes at $T < T_m$—are more resistant to the lytic action of deoxycholate than are bilayers in a fluid liquid-crystal state—as in EYL liposomes—as the likely result of the ease with which DOC molecules can be embedded into bilayers and disrupt their structures. Sagawa et al. (Sagawa et al., 1993) found the cytotoxicity of bile salts to be correlated with their hydrophobicity and that, in addition, phospholipid liposomal incorporation into bile salt micelles decreases with increasing fatty acyl chains saturation.

(b) As regards retention, the bilayer perturbation induced by sub-lytic concentrations of DOC is sufficient to cause the loss of entrapped solute. This is a frequent observation in studying the action of surfactants on liposomes and occasionally, also on cells. Thus, some authors have drawn a parallel between the leakage of cytoplasmic components and the interaction of deoxycholate with the lipid fraction of cell membranes. Shiao et al. (Shiao et al., 1989), who studied the action of various ionic and non-ionic detergents on human platelets, found that only deoxycholate resulted in membrane leakage prior to cell lysis. The action is believed to arise from transient holes formed during the packing and structural changes undergone by lamellae prior to solubilization of the bilayers.

(c) Regarding interactions between the acyl chains of the lipid matrix the perturbating effect of DOC on fatty acyl chains is obvious, even at low concentrations in the bulk aqueous phase, as judged through the steady-state fluorescence anisotropy measurements of the probe DPH. All three parameters derived from r_s measurements were consistent, so DOC: (1) alters acyl chain packing (see how T_m decreases in Fig. 6a); (2) decreases transition cooperativeness (see $\Delta T_{1/2}$ increase in Fig. 6b); and (3) diminishes the orderliness of acyl chains (as can be seen in Fig. 6c, $\Delta H^{\neq}$ decreases with increasing DOC concentration).

In summary, all that data show is the amphiphiles present in the biophase can commit to the effectiveness of the liposomes as drug carriers. The answer to this, in our opinion, is the use of a lipidic composition that ensures a tight packing of the acyl chains into bilayers without compromising the drug releasing ability of liposomes.

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References

Azumi, T., McGlynn, S., 1962. Polarization of the luminescence of phenantre. J. Phys. Chem. 37, 2413–2420.

Bartlett, G.R., 1959. Phosphorous assay in column chromatography. J. Biol. Chem. 234, 466–468.

Chantres, J.R., Elorza, B., Elorza, M.A., Rodado, P., 1996. Deoxycholate alters the order of acyl chains in FATVETs of DPPC. Study of the DPH steady-state fluorescence anisotropy. Int. J. Pharm. 138, 139–148.

Elorza, B., Elorza, M.A., Frutos, G., Chantres, J.R., 1993. Characterization of 5-fluorouracil loaded liposomes prepared by reverse-phase evaporation or freezing-thawing extrusion methods. Study of drug release. Biochim. Biophys. Acta 1153, 135–142.

Goñi, F.M., Urbaneja, M.A., Alonso, A., 1993. Effect of surfactants on the release of liposomal contents. In: Gregoriadis, G. (Ed.), Liposome Technology, vol. II. 2nd ed. CRC Press, Boca Raton, FL, pp. 261–273.

Helenius, A., Simons, K., 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta 415, 29–79.

Kovács, L., Zalka, A., Dobó, R., Pucsok, J., 1986. One-dimensional thin-layer chromatographic separation of lipids into fourteen fractions by two successive developments on the same plate. J. Chromatogr. B. Biomed. Appl. 382, 308–313.

Lasch, J., 1995. Interaction of detergents with lipid vesicles. Biochim. Biophys. Acta 1241, 269–292.

Lasch, J., Schubert, R., 1993. The interaction of detergents with liposomal membranes. In: Gregoriadis, G. (Ed.), Liposome Technology, vol II, 2nd ed. CRC Press. Boca Raton, FL, pp. 233–260.

Lichtenberg, D., Robson, R.J., Dennis, E.A., 1983. Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim. Biophys. Acta 737, 285–304.

Malloy, R.C., Binford, J.S. Jr, 1990. Enthalpy titration and solubilization of dimiristoyl-phosphatidylcholine vesicles with bile salts. J. Phys. Chem. 94, 337–345.

Paternostre, M.T., Roux, M., Rigaud, J.L., 1988. Mechanism of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by Triton X-100, octylglucoside, and sodium cholate. Biochemistry 27, 2668–2677.

Sagawa, H., Tazuma, S., Kajiyama, G., 1993. Protection against hydrophobic bile salt-induced cell membrane damage by liposomes and hydrophilic bile salts. Am. J. Physiol. 264, G835–G839.

Schubert, R., Beyer, K., Wolburg, H., Schmidt, K.H., 1986. Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. Biochemistry 25, 5263–5269.

Schubert, R., Beyer, K., Wolburg, H., Jaroni, H., Schmidt, K., 1986b. Membrane alterations induced by bile salts. Transient membrane holes as one possible mechanism of drug release from liposomes. In: Schmidt, K.H. (Ed.), Liposomes as Drug Carriers. Georg. Thieme. Stuttgart, pp. 61–70.

Shiao, Y.J., Chen, J.C., Wang, C.T., 1989. The solubilization and morphological change of human platelets in various detergents. Biochim Biophys Acta 980, 56–68.

Spink, C.H., Lieto, V., Mereand, E., Pruden, C., 1991. Micelle-vesicle transition in phospholipid bile salt mixtures. A study by precision scanning calorimetry. Biochemistry 30, 5104–5112.

Spink, C.H., Müller, K., Sturtevant, J.M., 1982. Precision scanning calorimetry of bile salt-phosphatidylcholine micelles. Biochemistry 21, 6598–6605.

van der Meer, B.W., van Hoeven, R.P., van Blitterswijk, W.J., 1986. Steady-state fluorescence polarization data in membranes. Resolution into physical parameters by an extended Perrin equation for restricted rotation of fluorophores. Biochim. Biophys. Acta 854, 38–44.

Walter, A., Vinson, P.K., Kaplun, A., Talman, Y., 1991. Intermediate structures in the cholate-phosphatidylcholine vesicle-micelle transition. Biophys. J. 60, 1315–1325.

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