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Deoxycholate alters the order of acyl chains in freeze-thaw extrusion vesicles of L-α-dipalmitoyl phosphatidylcholine: study of the 1,6-diphenyl-1,3,5-hexatriene steady-state fluorescence anisotropy

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Abstract

The destabilization of phosphatidylcholine bilayer membranes by the bile salt sodium deoxycholate (DOC) was studied from steady-state fluorescence anisotropy measurements. Freeze-thaw extrusion vesicles (FATVETs) of L-α-dipalmitoyl phosphatidylcholine (DPPC) composition were prepared by sequential extrusion through polycarbonate membranes and characterized for their overall inner volume, average size and size distribution, and lamellarity. Interactions between acyl chains in the lipid matrix, which reflect in the rotational diffusion motion in the 1,6-diphenyl-1,3,5-hexatriene (DPH) molecules, are perturbed by the presence of bile salt in the medium (even at low concentrations) below and above the main transition phase temperature of pure DPPC bilayers. Its effects on the lipid matrix are clearly reflected in the DPH steady-state fluorescence anisotropy (r_s) measurements. The resolution of r_s into its static (r_∞) and dynamic component (r_d) show that DOC affects both the amplitude and the velocity of DPH movements. However, at temperatures below the gel ↔ liquid crystal phase transition point, the static component (that reflects chain order) is more markedly affected than is the dynamic component (which reflects bilayer fluidity). Thus, at 31°C, angle θ, a measure of the amplitude of DPH oscillations, rises from 23° to 52° over the DOC concentration range from zero to 2.0 mM (equivalent to an effective molar ratio in the bilayer of $R_e^{25°} = 0.12$); at

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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; 5(6)-CF = 5(6)-carboxyfluorescein; Tris = tri(hydroxymethyl)aminomethane; 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; $< d_h >$ = mean hydrodynamic diameter; CMC = critical micellar concentration; D_t = total detergent concentration in the medium; D_w = detergent concentration in the aqueous medium; D_b = detergent concentration in bilayer; R_e = effective [detergent]/[lipid] molar ratio; $R_e^{sol} = R_e$ at complete solubilization; $R_e^{sat} = R_e$ at the onset of the lamellar to micelle transition; r_s = steady-state fluorescence anisotropy; S.D. = standard deviation.

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45°C, however, it varies from 62° to 77°. These changes in the bilayer packing status may be responsible for the alteration in the retention ability of liposomal formulations of the cytostatic agent 5-fluorouracil at sub-solubilizing concentrations of deoxycholate (Elorza et al., *J. Pharm. Sci.*, 1996, in press).

Keywords: Deoxycholate; Surfactant-liposome interactions; Drug delivery systems; 5-Fluorouracil; Extrusion (prep. meth.); Fluorescence anisotropy; DPH; 'Wobbling-in-cone' model

1. Introduction

The use of liposomes for the transport and delivery of drugs provides a means for increasing the therapeutic index of some of these. Liposome-surfactant (detergent) interactions play a central role in liposome-mediated drug delivery mechanisms, which have been comprehensively reviewed (Lasch, 1995). 5-Fluorouracil (5-FU), a potent cytostatic agent used for 20 years in the treatment of solid tumours and dermal diseases, is a firm candidate for liposomal encapsulation with a view to its parenteral or topic administration in order to avoid some of its undesirable side-effects (particularly those affecting the gastrointestinal duct and bone marrow) (Chabner, 1982). While in vitro and in vivo experiments have provided encouraging results, there remain a number of problems to be solved in this respect, such as those arising from the instability of liposomes in the biophase (i.e. retention disturbances caused by some plasma components). In fact, some amphiphilic molecules in biological fluids can induce its release, so liposome-surfactant interactions play a crucial role in the mechanism by which the drug is released by lipid vesicles.

As we have stated elsewhere (Elorza et al., 1996), based on turbidity measurements, the bilayer membranes of freeze-thaw extrusion vesicles (FATVETs) of L-α-dipalmitoyl phosphatidylcholine (DPPC) (FATVETs(DPPC)) in a rigid gel state are more resistant to lysis by deoxycholate than are bilayers in a fluid liquid-crystal state. However, the effective molar ratios [detergent]/[phospholipid] necessary for the saturation of the bilayers for their complete solubilization, R_e^{sat} and R_e^{sol}, display the same values at 25°C and 50°C, respectively. It therefore appears that the sodium deoxycholate (DOC) molecules display less capacity for insertion between those of the DPPC when the molecules of the phospholipid are densely packed. Thus the partition coefficient in the range of sub-solubilizing R_e is almost three times smaller at 25°C than at 50°C. The values of R_e^{sat}, R_e^{sol} and K, corresponding to the FATVETs (EYL) at 25°C are practically the same as those found at 50°C for the FATVETs(DPPC). Hence, for a phospholipid analogous series, the partition coefficient K corresponding to a surfactant/liposome system likely depends primarily on the physical state of the bilayer.

Moreover, the retention properties of FATVETs(DPPC) were impaired at sub-lytic DOC levels; 5-FU leakage amounted to $R_e^{50\%} = 0.09$ (where $R_e^{50\%}$ is the effective detergent/lipid mole ratio that induces release of 50% of the liposomal content), which is somewhat less at R_e^{sat} (1.2–1.4). This may be the result of the packing and structural changes undergone by the lamellae prior to solubilization of the bilayers.

The aim of this work was to derive information on the destabilizing action of the DOC ion on phospholipid bilayers. For this purpose, we studied its effects on the packing state of hydrocarbon chains of FATVET liposomes of dipalmitoylphosphatidyl choline composition by monitoring the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) molecules imbibed in the bilayer and by analysing the results in terms of its static and dynamic components, according to the 'wobbling-in-cone' model by Kinosita and colleagues (Kawato et al., 1977; Kinosita et al., 1977).

2. Materials and methods

2.1. Lipids and other reagents

Lyophilized L-α-dipalmitoyl phosphatidylcholine (DPPC) 99% pure, DOC, 5-FU, and the fluorescent probe DPH were supplied by Sigma.

5(6)-Carboxyfluorescein (5(6)-CF) was purchased from Eastman Kodak.

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 N_2 atmosphere at −20°C. All were titrated according to Bartlett (1959). Deoxycholate was recrystallized twice from hot ethanol. DPH was stored as a tetrahydrofuran (THF) solution in the dark. The polycarbonate filters used were purchased from Nucleopore. All other reagents were analytical-grade chemicals and bidistilled water was used throughout.

2.2. Preparation of vesicles

Multilamellar vesicles (MLVs) were formed from dried lipid films by resuspension in an aqueous buffer consisting of 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 10 μmol/ml in all preparations. The osmolality of the solutions was measured with a digital cryo-osmometer (Knauer).

FATVETs were prepared by subjecting MLVs to five freeze-thaw cycles in liquid nitrogen and warm water (60°C), followed by sequential cyclic extrusion through two stacked polycarbonate filters of 0.2 μm (5 cycles) and 0.1 μm pore size (10 cycles) at 55 × 10^5 Pa using an extruder (Lipex Biomembranes) jacked at a thermostating bath. Extrusion was performed at 55°C for FATVETs (DPPC) (T_m = 41°C for MLVs).

2.3. Characterization of dispersions

Vesicle dispersions were characterized based on their internal volume, V_i, average vesicle size and size distribution, and lamellarity. The overall internal volume of the dispersions was measured by using 5(6)-CF as label. The average V_i value (expressed in litres of encapsulated water per mole of phospholipid) and standard deviation obtained in six individual experiments was 1.70 ± 0.08 l/mol. The z-average hydrodynamic diameter of the vesicles $< d_h >$ and the size distribution of their suspensions were determined by photon correlation spectroscopy (PCS) using a Malvern Zetamaster-S submicron particle analyser. FATVETs formulations were found to be monodisperse (polydispersity index = 0.06), with an average diameter of $< d_h >$ = 92 ± 7 nm. Lamellarity was estimated from Mn^{2+} quenching of ^{31}P-NMR signal arising from the outward-facing head groups of the phospholipids in the vesicles. Measurements were performed at 25°C using a Bruker AXM-500 spectrometer. The average of three measurements shows the percentage of the external surface area is 48 ± 3%, so FATVETs (DPPC) used in this study are predominantly unilamellar.

2.4. Fluorescence anisotropy measurements

The variation of the steady-state anisotropy of DPH imbibed in lipid bilayers as a function of temperature and the effect of DOC on it were monitored on a Perkin-Elmer MPF-44A spectrofluorimeter as described elsewhere (Chantres et al., 1986). Briefly, 3 ml of FATVETs(DPPC) suspension containing 0.5 μmol/ml phospholipid were placed in a cuvette of 10 mm light pathlength. Next, 2 μl of DPH stock solution in THF were added (mole ratio of probe to phospholipid was 1:500). The sample was then allowed to stand at a temperature above the transition temperature of the phospholipid for 1 h in order to ensure equilibrium in probe distribution. All fluorescence emission measurements were corrected for light scattering (less than 1% in every case) by using a blank consisting of 3 ml of vesicle suspension containing no DPH, but rather 2 μl of THF. Samples were periodically shaken and their temperature maintained to within ± 0.1°C by means of a circulating water bath. The temperature was measured inside the cuvette by using a customized thermistor located immediately above the emission beam. The fluorescence signal and sample temperature were displayed via a digital millivoltimeter. The sample was maintained at equilibrium for 5 min before any measurements were made at each temperature. Samples were excited with vertically polarized light of λ_{ex} = 360 nm and emitted light analysed at λ_{em} = 430 nm. A

bandpass of 10 nm was used for both the excitation and emission beam, in addition to a 390-nm cut-off filter. Steady-state anisotropy values were determined 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 $G = (I_{VH}/I_{HH})$ is the Azumi-McGlynn factor (Azumi and McGlynn, 1962).

3. Results and discussion

The critical micelle concentration (CMC) of deoxycholate in the aqueous medium used was determined by using the fluorescent dye method. For this purpose, the fluorescence intensity I_f of solutions containing 1.0 μM DPH and increasing DOC concentrations was recorded. Extrapolation of the straight segment of the I_f (λ = 430 nm) vs. DOC concentration plot (Fig. 1) allowed us to estimate a CMC value of 2.7 mM, consistent with previously reported results (Helenius and Simons, 1975; Roda et al., 1983; Lasch and Schubert, 1993).

DPH is a rod-like molecule whose absorption and emission dipoles are almost parallel and lie along the principal molecular axis, which is perpendicular to the plane of the bilayer. The middle position of this probe in the bilayers is equal to that of the segment C_{10}–C_{11} of the hydrocarbonated phospholipid chains. Thus, the movements of the DPH molecules reflect those of the chains, and any modification of these movements (i.e. induced by the incorporation of a foreign molecule from the aqueous phase) will be seen in the fluorescence anisotropy of the DPH. Fig. 2 shows the influence of temperature on the steady-state fluorescence anisotropy, r_s, of DPH in the absence (Fig. 2A) of DOC and some examples (Fig. 2b–f) of its alteration in the presence of DOC in the medium.

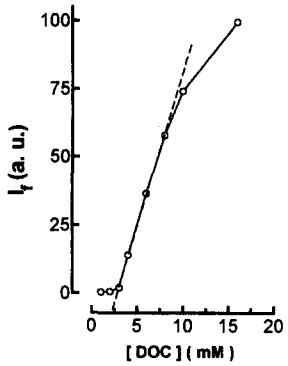

Fig. 1. Determination of CMC of deoxycholate in aqueous medium: intensity of the fluorescence signal of DPH (1 μM, λ_{ex} = 360 nm) as function of DOC concentration. From the extrapolation of lineal range results a CMC value of 2.7 mM. All measurements have been carried out at room tempearture.

In Fig. 2 the horizontal arrows indicate the temperature corresponding to the transition of the gel ↔ liquid crystal phase T_m in each sample. The vertical arrows indicate the positions corresponding to the values of T_R of 0.85 and 1.15 in each case, being $T_R = T/T_m$. That is to say, T_R here has the meaning of a 'reduced temperature', so that values equal to T_R correspond to states equally distanced from the average point of transition.

The effect of surfactant on bilayer integrity depends on the 'effective' molar ratio of detergent/phospholipid in the bilayer (R_e) and not on the 'total' molar ratio (R_t), R_e being defined as $R_e = D_b / PL$ and R_t as $R_t = D_t / PL$ with $D_t = D_b + D_w$. A partition coefficient is also defined as $K = R_e / D_w$. In a previous study (Elorza et al., 1996) we found that the value of R_e corresponding to the start of the solubilization of the bilayers, R_e^{sat}, (i.e. with the bilayers saturated by the deoxycholate) of FATVETs(DPPC) is 0.3 both at 25 and at 50°C. In turn, the R_e value corresponding to the completion of bilayers solubilization, R_e^{sol}, lies between 1.2 and 1.4 at both temperatures. Furthermore, the same results were obtained with FATVETs(EYL) at 25°C. This means that R_e^{sat} and R_e^{sol} are independent of the physical state of the bilayers. However, this factor certainly affects the partition coefficient K that for FATVETs(DPPC) at 25°C is 0.07 mM^{-1} and at 50°C is 0.16 mM^{-1}, and for FATVETs(EYL) at 25°C is 0.21 mM^{-1}. The partition coefficient K,

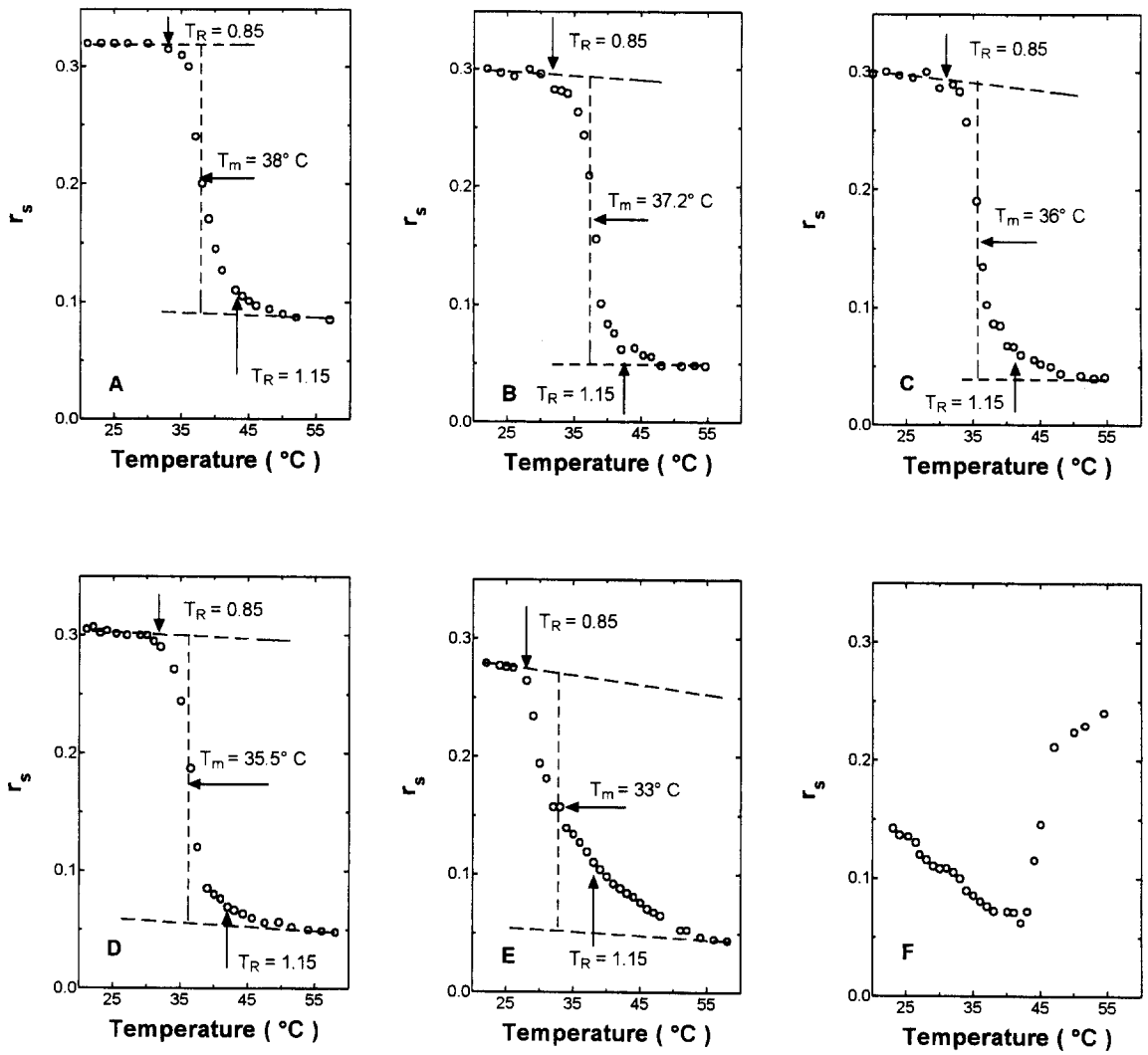

Fig. 2. Variation in profiles of steady-state fluorescence anisotropy, of DPH vs. temperature in FATVETs(DPPC) in function of the total concentration of DOC in the medium. (A) Without DOC; (B) 0.4 mM DOC; (C) 0.8 mM DOC; (D) 1.0 mM DOC; (E) 2.0 mM DOC; (F) 10.0 mM DOC. Horizontal arrow indicates the estimated transition temperature in this systems. The vertical arrows indicate the positions coresponding to the values of a 'reduced temperature', T_R, of 0.8 and 1.15 in each case, being $T_R = T/T_m$.

as it has been defined here only has significance in sub-solubilizing DOC concentrations range. R_e can be estimated, within that range, for any sample of liposomes by the relation:

$$R_e = \frac{D_t}{(\frac{1}{K} + PL)} \quad (2)$$

deducible from the previous expressions. From this point, DOC concentration corresponding to the results depicted in Fig. 2 are equivalent to an effective molar ratio $R_e^{25°}$ between 0.02 (Fig. 2B) and 0.6 (Fig. 2F), respectively. 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 derived from the r_s vs. T profile. The transition vanished by a DOC/phospholipid

effective mole ratio of 0.6, beyond which the r_s vs. T profile departed from the usual 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 at 25°C of 0.3 (results not shown).

In the bilayer, the restrictions imposed by the environment to the free rotational movement of the DPH molecules eliminate some of the possible orientations which contribute to the depolarization of the fluorescent emission. To this factor are added the dynamic conditions of the medium, which control the speed with which the probe performs its movements. These aspects mean the anisotropy is resolved into a stationary state in two components, one static (r_∞) and the other dynamic (r_d):

$$r_s = r_\infty + r_d \tag{3}$$

According to the 'wobbling-in-cone' model proposed by Kinosita and colleagues (Kawato et al., 1977; Kinosita et al., 1977) the movement of the DPH is restricted by the components of the bilayer lipid matrix, there being a relation between r_∞ and the aperture angle θ of the cone around the normal to the plane of the bilayer, whose expression is:

$$\left(\frac{r_\infty}{r_0}\right) = \left[\left(\frac{1}{2}\right)\cos\theta(1+\cos\theta)\right]^2 \tag{4}$$

where r_0 is the initial value limit of the anisotropy. In this study we have adopted the value $r_0 = 0.4$ for the DPH. Subsequently to the theory of Kinosita et al., Heyn and Jähnig (Heyn, 1979; Jähnig, 1979) established the relation between the parameter of order S and r_∞:

$$\left(\frac{r_\infty}{r_0}\right) = S^2; \qquad 0 \le S \le 1 \tag{5}$$

so, r_∞ is related to the degree of order of the hydrocarbonated chains of the bilayer. The works of van Blitterswijk et al. (1981), Pottel et al. (1983) and van der Meer et al. (1986) have shown the possibility of deriving the value of r_∞ from the measured steady-state data, r_s, by an empirical relationship applicable to a variety of artificial and biological membranes labelled with various probes. That relation is:

$$r_\infty = r_0 r_s^2/(r_0 r_s + (r_0 - r_s)^2/m) \tag{6}$$

the parameter m, which has a positive value, expresses the difference between the rotational diffusion of the probe in the bilayer and that occurring in an isotropic reference oil. For DPH van der Meer et al. (1986) found the value m = 1.7 in DPPC bilayers between 5 and 60°C. This value is the one we have used.

Fig. 3 shows the variation of static r_∞ (Fig. 3a) and dynamic r_d (Fig. 3b) components of r_s in function of temperature, in the absence and presence of 2.0 mM DOC (R_e = 0.12 at 25°C). Both r_∞ and r_d are affected. Before the transition of the gel ↔ liquid crystal phase takes place, the static component has decreased, while the dynamic component has increased. This decrease and increase in r_∞ and r_d are, in each case, the same at 25°C as at the temperature which marks the start

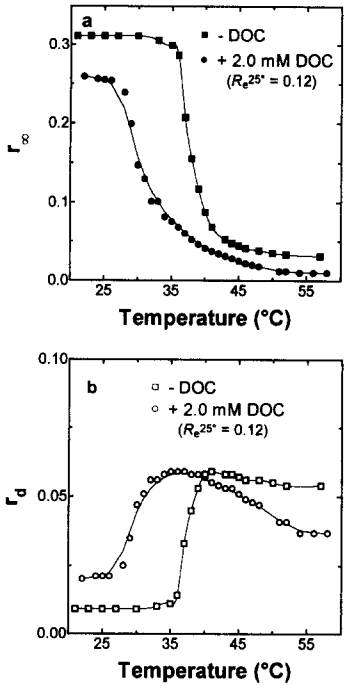

Fig. 3. Profiles of components of the steady-state fluorescence anisotropy, r_s. (a) Static, r_∞, and (b) dynamic, r_d, as function of temperature, in: (□) absence of DOC and (○) presence of 2.0 mM DOC in the medium, corresponding to an effective molecular ratio detergent/phospholipid into bilayer (R_e) of 0.12, at 25°C.

of the transition; this can be interpreted as meaning that the partition coefficient K does not change significantly in that temperature interval. In the temperature interval in which the gel ↔ liquid crystal transition takes place, the presence of DOC provokes the decrease of r_∞ and the increase of r_d. When the transition is complete, the values of r_d begin a decrease which places them below those obtained in the absence of surfactant, while those of r_∞ in the presence of DOC are still less than those obtained in its absence. It is also interesting to observe that once the transition is completed, in the absence of DOC, r_∞ and r_d are practically constant, while in the presence of the surfactant both values maintain their tendency to decrease, at least until 55°C. Of the results shown in Figs. 2 and 3, it can be deduced that the incorporation of deoxycholate into the bilayers of DPPC provokes a perturbation of the interactions between the chains which causes (a) a decrease in the temperature at which the gel ↔ liquid crystal transition takes place, and (b) an increase in the temperature interval in which the transition occurs. As a consequence, there is an increase both in the amplitude and in the speed of the Brownian rotation movement of the DPH molecules.

Fig. 4 shows the variation of r_s (Fig. 4a), r_∞ (Fig. 4b) and r_d (Fig. 4c) as a function of the effective molar ratio of surfactant/phospholipid in the bilayer R_e, below and above the transition temperature of each system, T_m. The interval of values of R_e finalizes at 0.3 corresponding to the saturation of the bilayers by the surfactant (Elorza et al., 1996) and marks the start of the solubilization of these bilayers and the appearance of mixed micelles.

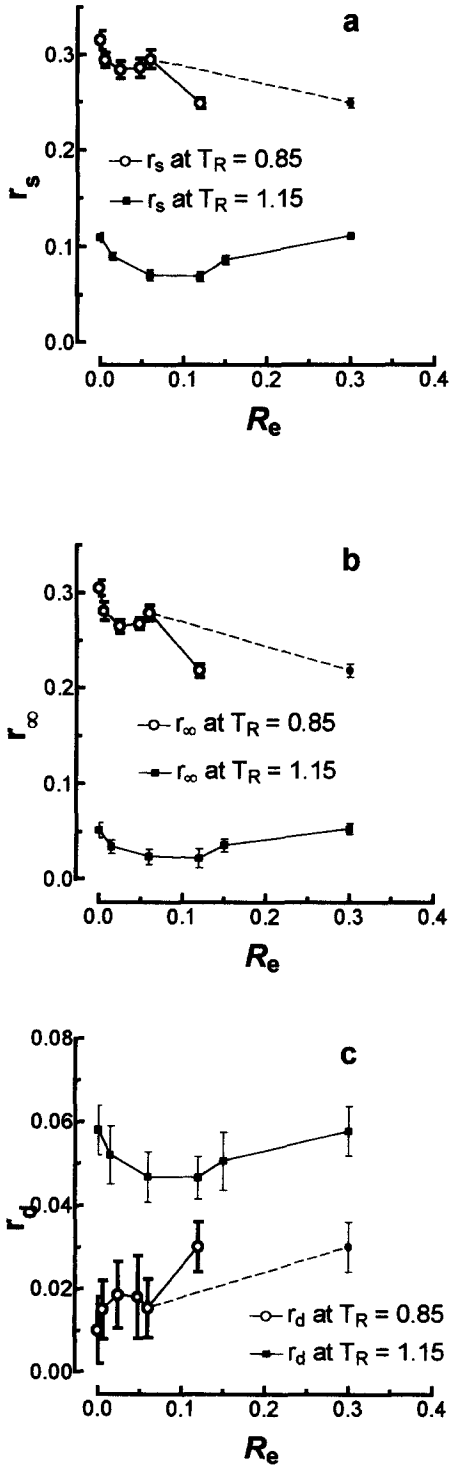

Fig. 4. Variation of r_s, r_∞ and r_d as function of the 'effective' molar ratio, detergent/phospholipid into bilayers, below (T_R = 0.85) and above (T_R = 1.15) of the transition temperature for each systems. Continuous line joints points calculated through a partition coefficient K = 0.06 mM^{-1}. Dashed line joints the point calculated through a value of K = 0.16 mM^{-1} (see text for more explanations). Bars symbolize the S.D. from six individual experiments.

For a correct comparison, the data must belong to states corresponding to the bilayer. We have therefore chosen to compare values of r obtained at the same relative temperatures ($T_R = T/T_m$) as at their respective transition temperatures. The results shown in Fig. 4a–c correspond to the 'reduced temperatures' $T_R = 0.85$ and $T_R = 1.15$ (approximately equivalent to $T = T_m \pm 5$°C). The values of R_e were calculated by means of Eq. (2). The value of the partition coefficient K used for the data corresponding to $T_R = 0.85$ was 0.06 mM^{-1} and for those corresponding to $T_R = 1.15$ was 0.16 mM^{-1}. We established these values by turbidity measurements performed at 25°C and 50°C on FATVETs(DPPC) (Elorza et al., 1996). As can be seen in Fig. 2a–d, $T_R = 0.85$ (arrows downward) marks the end of the gel phase, while $T_R = 1.15$ (arrows upward) corresponds very approximately to the end of the transition, when the bilayer is in the mesophase of liquid crystal. When the total concentration of DOC in the medium is 2.0 mM (Fig. 2E) the situation is somewhat ambiguous due to the wide temperature interval covered by the phase transition. Thus, when $T_R = 0.85$ the transition has started and when $T_R = 1.15$ it has not yet finished. For this reason in Fig. 4a–c we have also shown the value of r obtained at $T_R = 0.85$ in relation to the value of R_e calculated using the value of $K = 0.16$ mM^{-1} (point joined by dotted line).

At 31°C and concentrations below 1.0 mM (i.e. $R_e^{25°} = 0.06$) the action of the surfactant on the components of r_s is small. From 2.0 mM (i.e. $R_e^{25°} = 0.12$) both components undergo considerable changes, until their values equal those found at 45°C. This is due to the fact that from this concentration the transition starts at $T \leq 28$°C ($T_m \leq 33$°C), it being probable that in the most 'fluid' condition of the bilayer, the incorporation of the surfactant increases considerably. We have found (Elorza et al., 1996) that the partition coefficient governing the distribution of deoxycholate between the lipidic phase and the aqueous phase depends basically on the physical state of the bilayer. Thus a value of $K = 0.21$ corresponds to the partition of DOC between bilayers of egg-yolk phosphatidylcholine and the aqueous

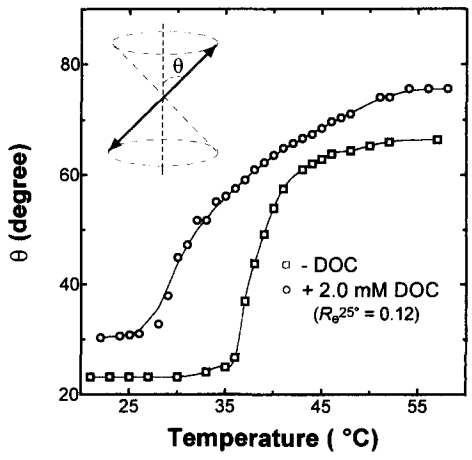

Fig. 5. Profiles of the 'wobbling-in-cone' angle, θ, vs. temperature, in function of the concentration of DOC in the medium. (□): without DOC, and (○): 2.0 mM DOC.

medium (Tris saline buffer, pH 7.4, 290 mOsm) at 25°C, which is practically the same as that found in the partition between bilayers of DPPC and the same aqueous phase at 50°C, $K = 0.16$. At 45°C, with the bilayers of the FATVETs(DPPC) in liquid crystal phase, the presence of DOC apparently

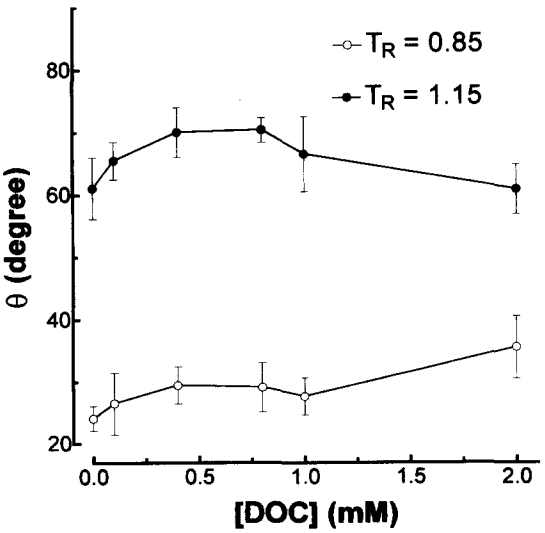

Fig. 6. Profiles of the 'wobbling-in-cone' angle, θ, vs. the total DOC concentration in the medium, below ($T_R = 0.85$; ○) and above ($T_R = 1.15$; ●) of the transition temperature for each system. Bars represent the S.D. from six individual experiments.

does not modify r_∞ and only slightly decreases r_d; that is to say, there is no 'condensing' effect similar to that induced by cholesterol, which without affecting r_d significantly decreases the values of r_∞ (Kawato et al., 1978; Kinosita and Ikegami, 1984).

Fig. 5 depicts the variation of the 'wobbling-in cone' angle θ of DPH molecules imbibed in FATVETs(DPPC) bilayers as a function of temperature, in the absence and presence of 2.0 mM DOC. When there is no surfactant in the medium θ remains constant at a value of $\theta \approx 23°$ at $T < (T_m - 2°C)$ and in a value $\theta \approx 67°$ at $T > (T_m + 2°C)$; the entire change occurs in the small interval of $T_m \pm 2°$. In the presence of 2.0 mM DOC (i.e. $R_e^{25°} = 0.12$) θ remains constant at a value of $\theta \approx 30°$ until $T \approx 28°C$, after which it varies progressively (more abruptly at the beginning) until at $T \approx 55°C$ it reaches the value of $\theta \approx 77°$. Fig. 6 shows the influence of total surfactant concentration on θ values at 31°C and 45°C, in the range equivalent to $0 < R_e^{25°} \leq 0.3$. While the bilayers are in the ordered gel phase the presence of DOC can be observed due to a slight increase in θ which, however, is not very sensitive to the increase of the concentration of the surfactant in the medium, probably as a result of the difficulty experienced by its molecules in incorporating into the bilayer in these conditions. When the transition is triggered as a consequence of the destabilizing action of the surfactant on the interactions between the phospholipid chains, θ increases at the same time as more and more DOC molecules are incorporated into the bilayers, which in turn promotes a greater freedom of movement in the chains which is reflected in a progressive increase of θ. At 45°C, with the bilayers in mesophase of liquid crystal and a greater facility for the DOC molecules to access its interior, (to judge from the partition coefficient value K at 50°C) the parameter θ appears more sensitive to the changes in the concentration of the surfactant, although the interval of variation does not exceed 10°. When the total concentration of deoxycholate in the medium is 5 mM (i.e. $R_e^{25°} = 0.30$) the lipidic phase is saturated by bile salt and the values of θ obtained at 31°C and 45°C practically coincide.

4. Conclusions

(a) As seen by the DPH molecule, even at low $[DOC]_{total}$, the perturbating action of surfactant on fatty acyl chains is obvious. The tendency of DOC to be embedded normally into the surface of bilayer membranes and become inserted among phospholipid molecules (Saito et al., 1983), supports this hypothesis. These effects on lipid matrices are clearly reflected in steady-state fluorescence anisotropy measurements of DPH.

(b) The bilayer perturbation induced by DOC at sub-solubilizing levels likely promotes packing and structural changes in the bilayer through which small molecules, such as 5-FU, can be leaked freely from the inner aqueous core of the liposomes, prior to solubilization of the bilayers.

The phospholipid bilayer is an anisotropic medium and the incorporation of a foreign molecule into its structure from the aqueous phase cannot be contemplated as a macroscopic partition phenomenon. Hence, the amount of surfactant that a phospholipid bilayer can accept depends on the packing state of the phospholipid molecules (acyl chains with head polar groups), the chemical structure of the surfactant and the surfactant-phospholipid interactions within the bilayer. Disposition of deoxycholate molecules may involve the introduction of polar hydroxyl groups in positions 3 and 12 into the hydrophobic core of the bilayers, which will have a destabilizing effect on DOC-phospholipid mixed vesicles.

In summary, the stability of liposomal formulations in relation to amphiphiles present in physiological fluids can be improved by ensuring that acyl chains in bilayers are tightly packed; this can be accomplished by using phospholipids of a high T_m and/or including cholesterol in the formulation. In any case, this should not compromise the drug releasing ability of liposomes, so bilayer stability and permeability should always be carefully balanced.

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