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Effects of flunitrazepam on the L_α-H_{II} phase transition of phosphatidylethanolamine using merocyanine 540 as a fluorescent indicator

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Abstract

Previous studies of our group demonstrated that flunitrazepam is a lipophilic drug capable of interacting with membranes through a partition equilibrium phenomenon. Its localization at the phospholipid polar head region could explain the decrease in the size of dipalmitoylphosphatidylcholine (dpPC) vesicles, through a mechanism that involves the increment in the relative volume of this region with a subsequent increase in the vesicle's surface curvature. In the present work, we investigated if flunitrazepam can affect the L_α-H_{II} phase transition of phosphatidylethanolamine through a similar mechanism. This study was approached by using merocyanine 540, a dye sensitive to the molecular packing of membrane lipids. A detailed analysis of merocyanine absorption and fluorescence emission and excitation spectra was performed. The results indicated that the fluorescence emitted came mainly from the monomeric form of merocyanine and that it resulted a good indicator of this phase transition, as was previously described. Flunitrazepam did not affect significantly the onset of the phase transition but showed a tendency to diminish the dye fluorescence emission intensity, which could involve a lower partition of merocyanine in the vesicles. Moreover, the results suggest that this drug produced a delay in the completeness of the phase transition and a decrement in the cooperativity of this phenomenon.

Keywords: L_α-H_{II} phase transition; Phosphatidylethanolamine; Flunitrazepam; Merocyanine 540; Absorption and fluorescence spectra

1. Introduction

Benzodiazepines (BZDs) are a group of neuroactive drugs widely administered due to their hypnotic, muscle relaxant, anxiolytic, and anticonvulsant effects. Flunitrazepam (FNT) is a BZD mainly used as a night-time hypnotic and it is commonly applied to the induction of anesthesia in surgery [1]. This kind of drugs affects neurotransmission by interacting with specific membrane-integral proteins [2,3]. However, due to its hydrophobicity, they can also interact non-specifically with other membrane components as lipids or non-receptor membrane proteins [4,5].

Non-specific interaction between membrane and lipophilic drugs can be described either by a Langmuir adsorption isotherm [6] or by a partition equilibrium model [7]. This latter model describes more adequately the type of BZD–membrane interaction, as was previously demonstrated by our laboratory, indicating that these drugs incorporate in the membrane as an integral part of the bilayer [8,9]. We showed that FNT and other BZDs partitioned within artificial phospholipid membranes and located at the polar head group region [9–11]. This interaction leaded to an increment of the relative volume of the polar head group region and to a decrease in the thermodynamic stability of the self-aggregating structure, which was forced towards an increase of its surface curvature. According to this rationale, it was proven that FNT was able to diminish the diameter of dipalmitoylphosphatidylcholine (dpPC) vesicles [11,12]. We also found that these phenomena could be transduced into changes in the curvature of membranes from natural origin [13].

It is known that the packing of lipids in membranes depends on thermodynamic factors coupled to the molecular geometry. Phosphatidylethanolamine (PE) has a small po-

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lar head group and two hydrocarbon chains that occupy a relative large volume. This inverted cone-like geometry favors the self-aggregation of PE in non-bilayer inverted micellar structures or hexagonal II phases (H_{II}) [14]. It was reported that molecules, with a shape opposite to this one, could inhibit the lamellar (L_α)-H_{II} phase transition of PE. This was shown by Perillo et al. [15] using different gangliosides, which have a large cross-sectional area in relation to the length of the hydrocarbon chains [16]. In the present work, we wondered if the localization of FNT between the polar head of phospholipids in the membrane could affect the L_α-H_{II} phase transition in PE vesicles.

Merocyanine 540 (MER) is a chromophore particularly sensitive to changes in the polarity of the medium. Its optical properties depend strongly on the environment [17]. It has been described the ability of this dye to sense the molecular packing of lipid structures [18–20]. Langner and Hui [19], developed a method based in these properties of MER to report phase transitions, such as the L_α-H_{II} [21], by measuring its fluorescence intensity as a function of the temperature in the presence of lipid vesicles.

In the present work, we analyzed in detail the absorption and fluorescence emission properties of MER in PE as well as its behavior in different solvents and at different temperatures, in order to contribute to a better understanding of the MER fluorescence in lipidic environments. This information was applied to investigate the possibility that FNT could affect the topological and the dipolar organization of PE.

2. Materials and methods

2.1. Materials

FNT (7-nitro-1,3-dihydro-1-methyl-5-(2-fluoro-phenyl)-1,4-benzodiazepin-2-one) was a kind gift from Productos Roche (Buenos Aires, Argentina). MER (5-[(sulfonyl-2(3H)-benzoxazoylidine)-2-butenyldene]-1,2-dibutyl-2-thiobarbituric acid) was purchased from Sigma (St. Louis, MO, USA) and was used without further purification. PE transesterified from egg phosphatidylcholine (tPE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Water was bidistilled in an all glass apparatus. All other drugs and solvents used were of analytical grade.

2.2. Preparation of lipid dispersions

Vesicles were prepared by evaporating, under a N_2 flux, a chloroformic solution of phospholipid. The dry lipid was resuspended in water by repeating five consecutive cycles of 1 min of vigorous shaking at room temperature. The lipid dispersion was passed 15 times through a 0.1 μm cellulose filter using a mini-extruder Liposofast (Avestin Inc., Ottawa, ON, Canada), in order to obtain vesicles with a homogeneous size with diameters around 100 nm. The filtration process was done on ice to avoid the formation of H_{II} phase. The vesicles were always used immediately after their preparation and their use from stock solutions was totally avoided.

2.3. Absorption and emission spectra of MER

Absorption spectra were obtained using a Beckman DU 7500 (Fullerton, CA, USA) spectrophotometer equipped with a diode array detector and a thermostated cell holder. Experiments were done at 20 °C and the samples contained tPE vesicles (0.6 mg/ml final concentration, in water) with or without MER (0.75 μg/ml final concentration) solubilized in ethanol (0.25% (v/v) final concentration of ethanol in the sample). The absorbance values from the spectrum of the blank (sample without MER) were subtracted from the corresponding to the sample containing MER, in order to correct for the turbidity and the scattering effects of tPE.

Steady state fluorescence emission was measured with a Fluoromax-Spex-3 (Jobin Yvon Inc., Edison, NJ, USA) spectrofluorometer equipped with a thermostated cell holder. Emission spectra were done by measuring the fluorescence emission within a specified range of wavelength and exciting at a fixed wavelength. Excitation spectra were obtained by exciting within a specified range of wavelength and recording the fluorescence with the emission monochromator settled at a fixed wavelength. The samples contained tPE (0.05 mg/ml final concentration) in the absence (blank) or in the presence of MER (0.06 μg/ml). The latter was added to the sample from an ethanolic solution so that the final ethanol concentration was 0.25% (v/v). The samples were incubated at 23 °C or at 65 °C, according to the experiment.

2.4. Determination of L_α-H_{II} temperature transition

In these experiments, we followed the method described by Langner and Hui [21] with some modifications. Briefly, the MER was added to the vesicle solution at a final concentration described above for fluorescence spectra. In this work, the incorporation of MER to the sample was done using two different procedures:

(I) MER was added to the sample at the lowest temperature and the same sample was heated through all the temperature range (approximately 20–65 °C); the sample was incubated for 15 min at each temperature and then the fluorescence emission was determined.
(II) MER was incorporated to different samples previously maintained at each assayed temperature and incubated for 15 min before measuring.

The dye fluorescence emission at 585 nm (exciting at 540 nm) was determined and then corrected for the hypochromic effect of the temperature by using the fluorescence of MER in ethanol as a reference curve, according to [19]. Moreover, we did this correction by using two additional solvents with different dielectric constant (D) (dioxane and butanol) to evaluate which of them was the best to correct this effect.

3. Results and discussion

3.1. Dimerization equilibrium of MER in tPE

3.1.1. Absorption spectra

MER has the property to undergo an equilibrium between a monomeric and a dimeric form. These chemical species exhibit maximal absorption wavelengths, which are both different between one to another and sensitive to the polarity of the environment. In the presence of bilayers in fluid state, these absorption peaks are centered at 568 and at 530 nm for monomer and dimer, respectively [22].

Fig. 1 shows the absorption spectrum of MER in the presence of tPE vesicles at 20 °C. A peak centered at 569 nm, with a shoulder at 530 nm, is clearly appreciated. Thus, while the peak would correspond to the absorption of the monomeric form of MER, the shoulder would represent the absorption of the dimer [20]. Similar absorption bands were observed for MER in Triton X-100 micelles [23] and in dpPC vesicles in the liquid-crystalline phase [20], indicating similar dielectric properties of the environments sensed by MER in all these structures. The relative heights of the two absorption bands, that we obtained with MER spectrum in TPE (Fig. 1), resemble that found in Triton X-100, and denote a higher contribution of the monomer if it is compared with dpPC in gel phase. This suggests either a larger partition of this chemical form with respect to the dimer in the present vesicle system (fluid phase) or, more probably, a displacement of the dimerization equilibrium toward the monomer in the whole system. Taking into account that:

(i) the peak at 568 nm appears only in media with $D < 10$ [23];
(ii) the extinction coefficients of the monomer and the dimer at 569 nm is near 30 [24];
(iii) the values of the equilibrium of dimerization constant in a low polar environment, like multilamellar vesicles of dpPC, and in water are approximately 10 and $10^4\ M^{-1}$, respectively [20,25]; and
(iv) the total concentration of MER in water in the present study is near 1.5 μM,

it can be estimated that the ratio between the concentrations of the monomeric and the dimeric species (monomer/dimer) in the present experimental conditions are approximately 1000 in tPE bilayers and 0.33 in water.

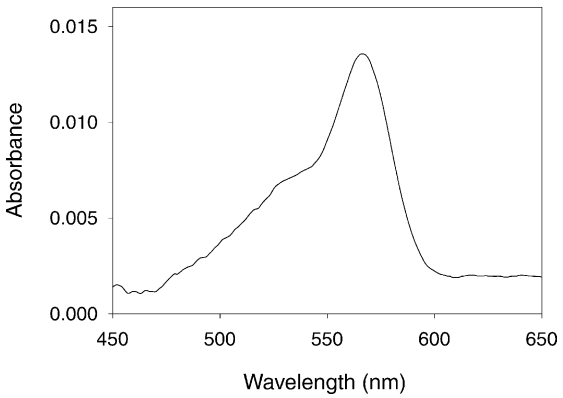

Fig. 1. Absorption spectrum of MER in the presence of tPE vesicles. The experiment was done at 20 °C. The final concentrations of MER and tPE in water were 0.75 μg/ml and 0.6 mg/ml, respectively. The curve corresponds to the absorption measurement of MER in presence of tPE minus the absorbance of a sample with only tPE.

3.1.2. Fluorescence spectra

It has been reported that, in a membranous system in a fluid state, the phenomenon of absorption at 568 nm by the monomer produces a characteristic fluorescence emission spectra with a peak centered at 585 nm, while the absorption at 530 nm by the dimer induces an emission spectra with a maximum at 630 nm [20]. Hence, the fluorescence emission spectra of MER were recorded at 23 and at 65 °C (below and above the L_α-H_{II} phase transition temperature reported for tPE) with an excitation wavelength of 540 nm (Fig. 2a) and 570 nm (Fig. 2b), corresponding to the approximated regions of maximal absorption of the dimer and the monomer, respectively. Both spectra exhibited a peak of fluorescence emission at approximately 585 nm (corresponding to the emission of the monomer) at both temperatures, even though at 65 °C the intensity of the fluorescence emission was lower due to the hypochromic effect of temperature (explained below). Conversely, no marked emission peak at 630 nm from the dimer was observed. In agreement with these results, the excitation spectra at 23 °C (Fig. 2c and d, respectively) showed only one peak near to 570 nm (due to the excitation of the monomer) even if the emission wavelength was set either at 585 nm or at 630 nm. The excitation spectrum at 65 °C, setting the emission at 630 nm, showed a batochromic displacement and lower intensity values with respect to that obtained at 23 °C. When the emission was settled at 585 nm no peaks were clearly seen. At both temperatures, FNT tended to reduce the fluorescence intensity of the peaks, as shown in all the graphs of Fig. 2. The emission and excitation spectra of MER at both temperatures, corresponding to the L_α and the H_{II} phases, respectively (Fig. 2), are in accordance with the previous analysis performed on the absorbance data and support the fact that the fluorescence emission measured at 585 nm (excitation at 540 nm) in the present system (tPE vesicles) comes almost exclusively from the monomer. The dimer is able to fluoresce significantly when it is partitioned in membranes in the gel phase but not in water or in fluid phases, or at least it does it with a lower intensity. This is due to the fact that both lipid phases stabilize the dimer in different energy states. A gel phase reduces the intramolecular movement of the dimer and its diffusional collisions with solvent molecules, which would increment the radiative process [20,26].

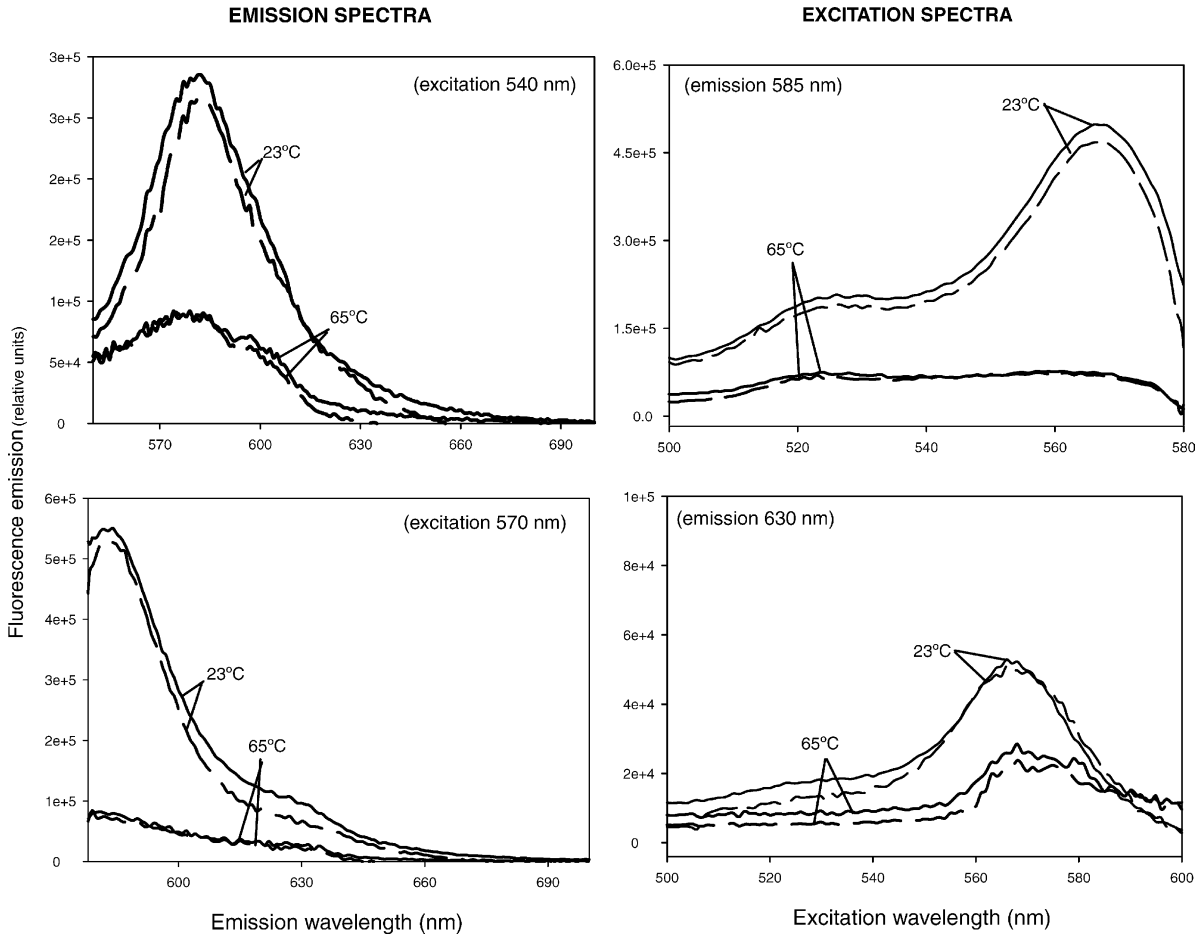

Fig. 2. Fluorescence spectra of MER in tPE vesicles. The experiments correspond to emission (a, b) and excitation (c, d) spectra setting, respectively, the excitation or emission wavelength indicated in each graph. The samples contained MER 0.06 μg/ml and tPE 0.05 mg/ml. The spectra were done at 23 °C or 65 °C as is indicated in the graph. Each value of the curves represents the mean of three determinations. Control: solid lines. With FNT 50 μM: dashed lines.

3.2. Temperature-induced quenching of MER fluorescence emission in isotropic media of different polarity

Fig. 3 shows the fluorescence emission (exciting at 540 nm) of MER in different solvents and at different temperatures. The results indicated that the emission intensity decreased as a function of the temperature in all the solvents assayed (see Fig. 4). Moreover, the lower the solvent polarity it was observed not only a reduction in the fluorescence but also an hypsochromic shift of the fluorescence maximum, at all the temperatures assayed. Thus, while the former behavior corresponds to a loss of quantum yield [19], the latter reflects the effect of the polarity of the medium where the MER was dissolved [23,27,28]. The D of the solvents used are: ethanol = 24.5, butanol = 17.5, and dioxane = 2.2 [29].

A straight line ($y = a + bx$) was adjusted to the values of fluorescence emission obtained as a function of temperature in each solvent (Fig. 4) and the results of the linear regressions were indicated in the right hand side of the graph. The corresponding slopes were used in the experiments that followed, in order to correct for the effect of the temperature on the fluorescence emission of MER in the presence of lipid vesicles (Fig. 5).

3.3. L_α-H_{II} phase transition of tPE in the absence and in the presence of FNT

The fluorescence emission of MER at 585 nm (excited at 540 nm) in the presence of tPE vesicles as a function of the temperature, using the procedure I or II (see Section 2) are shown in Fig. 5a and c, respectively. In the same figure, the graphs (b) and (d), correspond to the corrected values of fluorescence shown in graphs (a) and (c), respectively, using the regression lines obtained in Fig. 4 for each solvent, as explained above. In the curves obtained through the procedure I, the fluorescence emission remained almost constant up to about 58 °C; at this temperature the slope acquired positive values indicating the L_α-H_{II} phase transition point. A similar behavior was observed by applying the procedure II and using the correction obtained with dioxane; in this case the fluores-

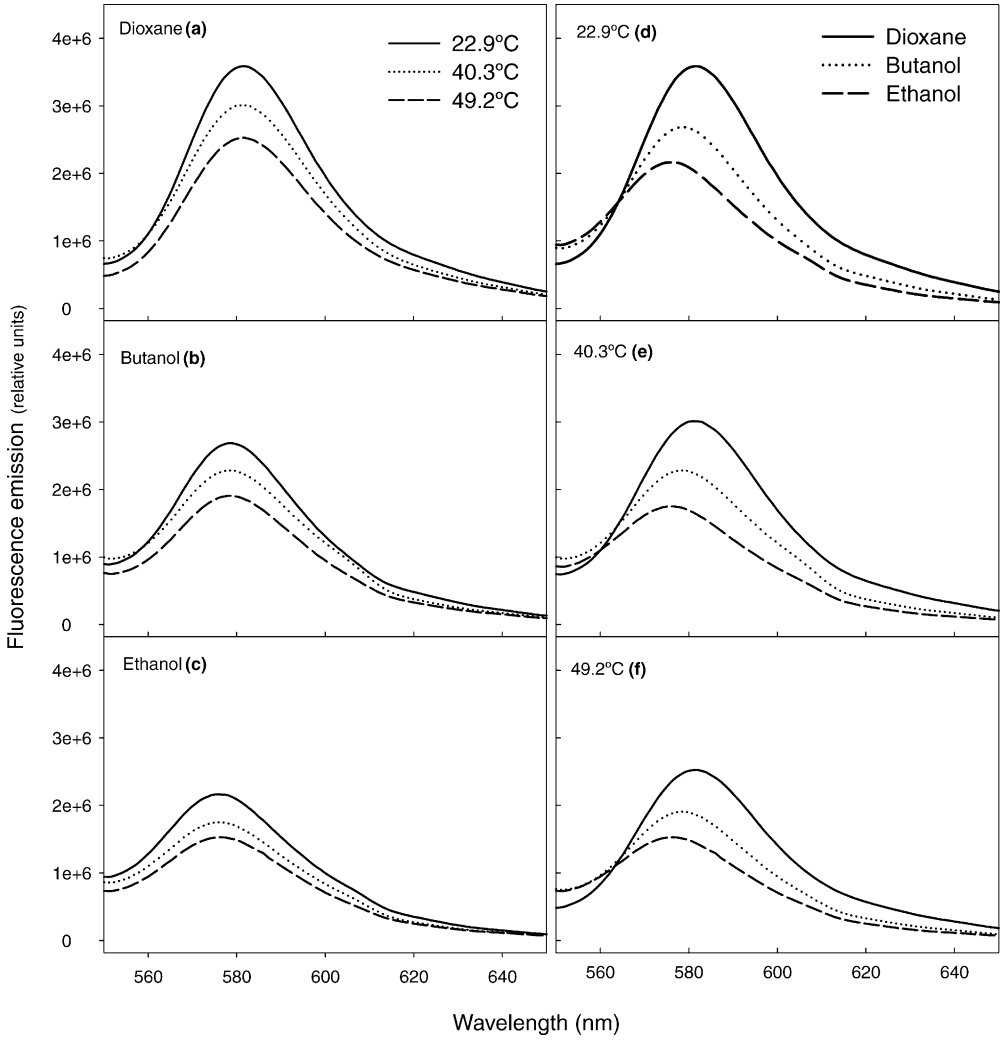

Fig. 3. Effect of the solution medium and the temperature on the fluorescence emission spectra of MER. The curves represent the emission spectra of MER (0.05 μg/ml) in dioxane (a), butanol (b), and ethanol (c) at different temperatures, and at 22.9 °C (d), 40.3 °C (e), and 49.2 °C (f) in different solvents. Excitation wavelength: 540 nm. Each value of the curves represents the mean of three determinations.

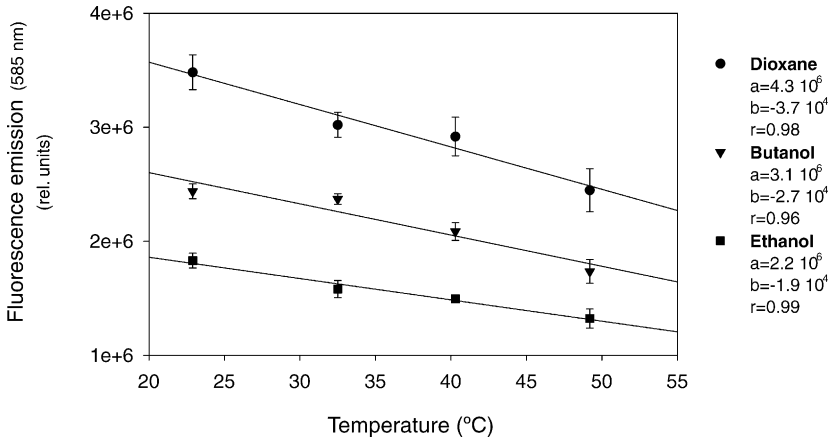

Fig. 4. Fluorescence emission of MER in different solvents as a function of temperature. The points correspond to the emission at 585 nm (excitation at 540 nm) of MER (0.05 μg/ml) as a function of temperature in dioxane, butanol, and ethanol, and were extracted from Fig. 3. Linear regressions were fitted to the data and the results are showed outside the graph; (a) ordinate; (b) slope. The bars represent the standard error of the mean (S.E.M.) of triplicates.

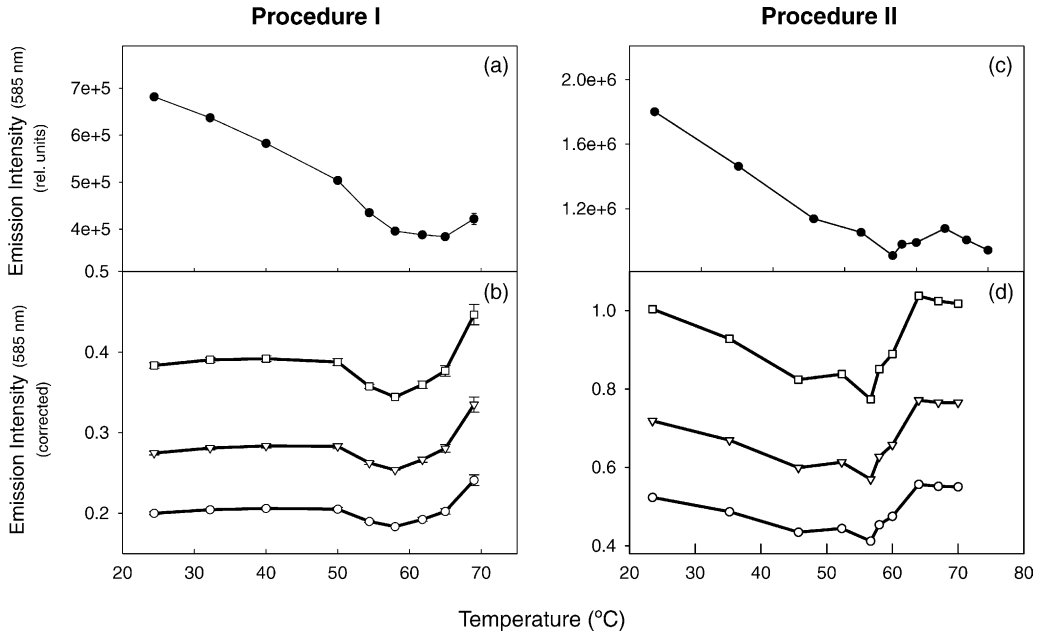

Fig. 5. Fluorescence emission of MER in presence of tPE vesicles. The values represent the fluorescence emission (a, c) and the corrected fluorescence emission (b, d) using the corresponding curve with dioxane (○), butanol (▽) or ethanol (□) from Fig. 4. Left panels (a, b) correspond to procedure I and right panels (c, d) correspond to procedure II. The bars represent the S.E.M. of triplicates. See details in Section 2.

cence intensity again showed an abrupt increment at about 58 °C.

The L_α-H_{II} phase transition temperature, reported for tPE vesicles by X-ray diffraction, is near 60 °C [21]. It was previously demonstrated that the correction of fluorescence eliminates the hypochromic effect of temperature and that the resultant values represent only the effect of the lipid molecular packing [21]. Thus, it is expected that the MER emission exhibits a steady behavior at the temperatures below 60 °C as well as an increment after reaching the L_α-H_{II} phase transition, indicating a decrease in the order of the lipid environment. From the three solvents used to correct the effect of the temperature on MER emission, dioxane let obtain the results closest to those previously reported, which strongly suggest that this solvent represents more adequately the environment where MER is located within tPE vesicles. In experiments using phosphatidylcholine liposomes, it was proposed that MER resides slightly above the glycerol backbone [18]. Nevertheless, other authors described that this dye is located in very low polar regions of membranes ($D \cong$ 4–9) [23,30], supporting the fact that the correction of the hypochromic effect of the temperature obtained with dioxane was better if compared with solvents of higher polarity. However, in order to use the method already described by Langner and Hui [21] and taking into account that the transition point is clearly indicated by the change of the sign in the slope, we used ethanol in the experiments that followed.

Within the range of temperatures where the fluorescence emission of MER in tPE remains stable, it is expected that the lipid system remains in an almost constant molecular packing condition. At higher temperatures, in the region just preceding the transition point, a decrease of the corrected MER emission is observed independently of the procedure used (I or II). This decrement could be understood by considering that the phase transition involves a substantial exposure of the hydrocarbon chains to water [31] and that the lower fluorescence intensity would be expected because the quantum yield of MER decreases if it is compared to that of membrane environment, due to the higher polarity of aqueous media (Fig. 4 and [17]).

Both procedures reported the same temperature for the L_α-H_{II} phase transition of tPE. Hence, similar results were obtained either if MER encounters the membrane when the temperature (and subsequently the molecular organization) has already been settled, or if it faces the membrane at low temperatures and it is present during the whole heating process. This behavior, would permit to employ the more practical procedure I to determine phase transition temperatures by using MER.

The effect of 50 μM FNT on the fluorescence emission of MER with tPE vesicles is shown in Fig. 6. This concentration of FNT had been proven as capable to affect the curvature properties of natural and artificial membranes as reported previously [12,13]. The analysis of the effect of FNT indicated that this drug did not affect significantly the onset of L_α-H_{II} phase transition temperature of tPE. However, it was evidenced a tendency to diminish the intensity of the fluorescence emission of MER, as was described in the Fig. 2 for the emission spectra, and an increment in the width of the downward peak. Both facts suggest that FNT delayed the completeness of the phase transition and that it decreased the cooperativity of the phenomenon, respec-

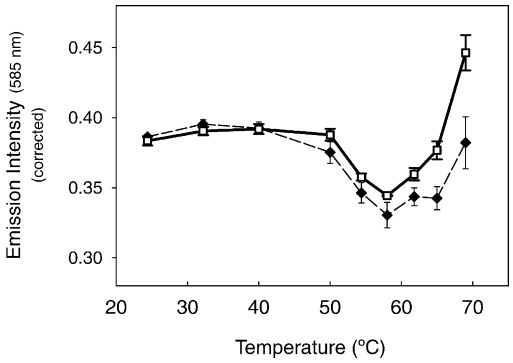

Fig. 6. Effect of FNT on the fluorescence emission of MER. The values represent the fluorescence emission of MER with tPE vesicles in the absence (control) (□) or in the presence of 50 μM FNT (◆), corrected by the corresponding curve with ethanol and using the procedure I. The control values correspond to those shown in Fig. 5b. The bars represent the S.E.M. of triplicates.

tively. This is more clearly observed in the experiments using the procedure I and at the higher temperatures. Taking into account that, at approximately neutral pH as the used in this work, MER presents a negative charge [32] and PE and FNT remain uncharged [8,10], it might not be expected an electrostatic influence on the partition neither of MER nor of FNT. At the MER–lipid molecular ratio used in this work (1:600 approximately) near to 60% of MER is partitioned in the membrane in fluid state [19]. Considering that FNT is a lipophilic drug able to interact with phospholipid membranes (partition coefficient ≅ 70, in a dpPC–buffer system [8]) and its location at the polar head group regions [10], it can be suggested that the lower intensity of the fluorescence emission of MER in the presence of FNT would be due to structural changes caused by the partition of this drug, which impairs the movement of MER out of the membrane. This interpretation was confirmed with other experiments (see below).

It was recently described for some PEs, that increasing temperature induces the increment in the polar head group area at the lipid–water interface and a decrease in the average lipid length and in the interlamellar water-layer thickness [33]. This fact could explain the lack of effect of FNT on the L_α-H_{II} phase transition temperature of tPE, perhaps because the increment of the polar head group area induced by the temperature makes negligible eventual additional structural effects induced by FNT in this polar region.

3.4. Hysteresis between the heating and cooling in the fluorescence emission of MER in an anisotropic medium

Fig. 7 represents the effect of the temperature increment (heating) and the subsequent decrement (cooling) on the intensity of fluorescence emission of MER, following the procedure I. In samples using ethanol as an isotropic medium, a significant hysteresis in the values determined along the heating–cooling cycle was observed (Fig. 7a). Nevertheless, when the ratio between the intensity of fluorescence emis-

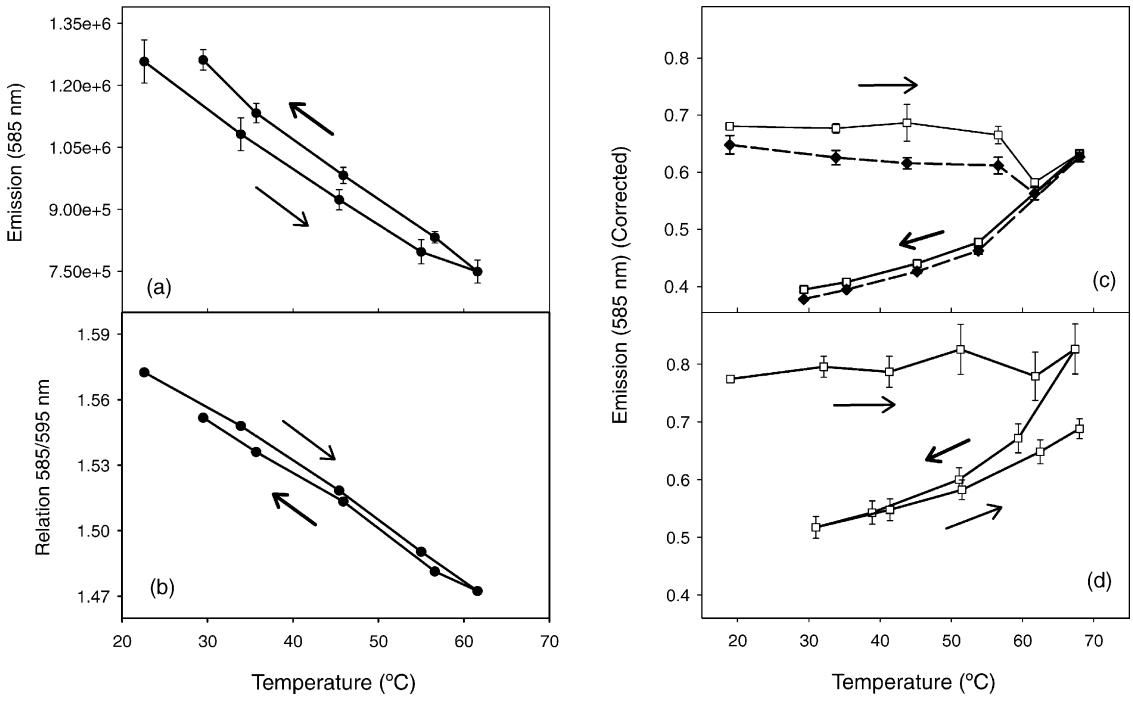

Fig. 7. Effect of heating and cooling cycles on the fluorescence emission of MER. Fluorescence emission of MER (0.05 μg/ml) at 585 nm (exc. 540 nm) in ethanol (a) or in the presence of tPE vesicles (c, d). (b): Represents the ratio between the emission at 585 and 595 nm of the same experiment that panel (a), calculated in order to avoid the effect of ethanol evaporation. (d): Corresponds to a separate sample submitted to a second heating process. In panel (c), the full diamond symbols correspond to samples in the presence of 50 μM FNT. The arrows indicate the heating or cooling process. The bars represent the S.E.M. of triplicates.

sion at 585 and 595 nm (585/595) was calculated, in order to avoid the effect of the solvent evaporation, the system showed a reversible behavior (Fig. 7b). In a tPE vesicle system, a significant difference between the corrected intensity values of the fluorescence emission of MER within the heating–cooling cycle was observed, either in the absence or in the presence of FNT (Fig. 7c). Contrary to what happened in an isotropic solvent, the difference between these pathways did not disappeared after calculating the 585/595 fluorescence ratio (not shown) indicating that it was not due to an effect of solvent evaporation but reflected a hysteretic behavior of this anisotropic system. Additionally, the path followed during a second heating process, was not the same as that in the previous cooling process (Fig. 7d). In tPE, the lower values of fluorescence emission corresponding to the cooling process, with respect to the first heating process, would reflect a lower partition of MER in the membrane and would not be due to changes in the environment where the dye is located. This last statement is confirmed by the fact that the position of the peaks in the emission spectra do not vary, independently of the temperature value and of the direction of the temperature change (results not shown). It is accepted that this kind of phase transitions typically exhibit hysteresis characterized by an apparent phase transition temperature upon heating higher than upon cooling [31]. During the cooling process of tPE vesicles, the transition point would tend to disappear or more probably to show a less sharp transition at lower temperatures (Fig. 7c and d). This behavior was observed in other phase transitions like the pretransition of dpPC [34] and likewise may reflect slow changes in the structure of water at the lipidic surface. Moreover, it stresses the importance of the procedure applied for the sample preparation, described in the experimental section, in order to detect the phase transition.

FNT would affect the accessibility of MER to the new position that became available during the heating process (Fig. 7c; note the lower fluorescence values in the presence of FNT within the region of the curve marked with the arrow pointing to the right). However, FNT could not displace MER from the positions reached at the highest temperatures (the fluorescence of MER in the presence and in the absence of FNT were superimposed during the cooling process showed in Fig. 7c).

4. Conclusions

- The fluorescence emission of MER partitioned in tPE bilayers corresponds only to the monomer emission.
- The presence of FNT induces a slight decrement in the fluorescence emission of MER in tPE probably by a reduction of MER partition due to the localization of FNT in the membrane.
- The L_{α}-H_{II} transition phase temperature determined by this method was approximately 58 °C. The presence of FNT did not affect significantly this value, but reduced the cooperativity and retarded the completeness of the phase transition.
- An important hysteresis process was observed in the fluorescence emission of MER during the heating–cooling cycle, probably due to a lower MER partition and/or a smother or absent phase transition. FNT affected MER access to the new available positions upon heating but could not displace MER from the positions already reached at the highest temperature.

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