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The interaction of D-propranolol and dimyristoyl phosphatidylcholine large unilamellar vesicles investigated by quasielastic light scattering and Fourier-transform infrared spectroscopy

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The effect of D-propranolol on dimyristoylphosphatidylcholine large unilamellar vesicles has been investigated by quasielastic light scattering and Fourier transform infrared spectroscopy. The results indicate that the drug interacts with the phosphate groups of the polar heads, shifts the phase transition point and alters the low temperature phase. A second transition has been observed, and the new phase of the vesicles induced by the presence of the drug might be an interdigitated one.

Keywords: dimyristoylphophatidylcholine; DMPC; model membrane; propranolol; local anesthetics; phase transition; quasielastic light scattering; FT-IR spectroscopy; interdigitation.

Introduction

In a cell membrane, the lipid matrix constitutes the skeleton in which the proteins and other constituents are embedded. It is well known that there is a relationship between the response to drug action and the lipophilic character of the drug [1]. The support of this relationship is that the drug must cross lipophilic membranes before reaching its receptor site. In order to obtain information about the drug-membrane interaction, it is interesting to know how the drug modifies the behavior of the phospholipidic matrix of the membrane.

In general, phospholipidic bilayers exist under two phases (the liquid-crystalline phase and the gel phase) depending on the temperature. Recently, a new phase has been discovered, the interdigitated phase obtained by the dissymetry of two acyl chains or induced by some agents such as proteins, ethanol, chlorpromazine or tetracaine [2—7]. The observed data are not yet numerous but sufficient to state some conditions for an agent to induce such a lipid phase: it must interact with the polar head at the aqueous interface and not penetrate deeply into the hydrophobic region to facilitate the interdigitation of the acyl chains of the two opposing monolayers. The interdigitated phase exists at low temperature as a particular gel phase. From the structural point of view, it is easy to conceive that in such a state, the thickness of the membrane becomes reduced, the membrane is highly packed and this may be related to the decrease of the passive permeability. Moreover, as a consequence, the average sectional molecular area per polar head as well as the area of the vesicle become increased. The interdigitation effect has been in-

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vestigated at a molecular level by Levin et al. [2] and Auger et al. [7] using Raman spectroscopy and high pressure infrared spectroscopy. At a more macroscopic level, the variation of the thickness has been shown by X-ray scattering [4] and we will show that the variation of the vesicle area induced by the interdigitation can be studied by quasielastic light scattering.

In this work we are interested in a particular drug, the propranolol, ((*R*)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol). This drug is used as an antagonist of the catecholamine receptor [8—12] at low concentrations (10^{-7} M) but at higher doses it displays a local anesthetic activity [13]. Pharmacologically it is known to have a "stabilizing" effect on biological membranes [10—12]. There are two enantiomers of the propranolol molecule and it is believed that the D-form is responsible for the stabilizing effect [14].

A local anesthetic prevents the sodium permeability across the membrane and some workers think that it interacts with the lipid bilayer and indirectly blocks the sodium conduction. Much has been written in order to elucidate this point of view [7,15]. McIntosh et al., by X-ray diffraction and Auger et al., by high pressure IR spectroscopy have shown that local anesthetics such as tetracaine induce an interdigitated phase in model membranes [4,7].

Concerning propranolol, more than 1000 works dealing with its clinical effects have been published [8—13,16,17]. Most of them are related to its receptor blocking activity which is a property of the L-form. Pharmacological properties related to the D-form and reported to its stabilizing effect are generally considered as unwanted effects (sleep disturbances, inhibition of enzymes embedded in membranes, depressing effect on ionic current in the heart) but others are benifical (reduction of essential tremor, inhibition of the bioactivation of thyroxine in hyperthyroidism) [18,19]. Few reports have been concerned with the physico-chemical behavior of propranolol. Those on its effect on the zeta potential [20,21], the fluidity [22] and the thermal behavior [23] of model membranes were the rare ones found.

In this paper we report the results of a study about the effect of D-propranolol on phospholipidic model membranes by FT-IR spectroscopy to locate the interaction and quasielastic light scattering, in a more macroscopic manner, to investigate the effect on the vesicle size. The results suggest that D-propranolol interacts mostly with the polar head of the phospholipidic bilayer inducing an interdigitated phase at low temperature.

Materials and Methods

Materials

Dimyristoyphosphatidylcholine (DMPC) was purchased from Sigma. The purity of the lipid was checked by thin layer chromatography and the lipid was used without further purification. D-propranolol, (*R*)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol (molecular formula shown in Fig. 5) was obtained from Aldrich (p*Ka*) = 9.4) [16]. Unilamellar vesicles (LUV) were prepared in 150 mM NaCl, 10 mM Tris—HCl, pH 8.3 aqueous buffers by the reversed phase evaporation method of Szoka and Papahadjoloulos, as previously described [24]. This technique allowed the obtention of unilamellar vesicles of size from 90 nm to 160 nm. By successive centrifugations large size vesicles of 160 nm in diameter were obtained, with a polydispersity factor of 0.15 to 0.20.

Lipid concentration was checked using the colorimetric method [25]. Preparations were stable within 10 days (same size, same polydispersity factor).

Propranolol solutions in the same buffer were added 12 h before measurements into vesicles samples in order to obtain the desired propranolol/DMPC ratio.

Quasielastic light scattering

Quasielastic light scattering (QLS) was performed on a photon beating spectrometer operating at right scattering angle. Correlation functions were obtained with a 128-channels correlator Malvern K 7032 interfaced with an Olivetti calculator which allows data analysis. Solutions were centrifuged and filled into cylindrical cells of 10 mm in diameter.

Cells were fixed into a thermostated bath. The temperature of the samples was controlled to 0.1°C and temperature measurements were obtained at discrete intervals with a waiting time of 20 min. Each result is an average of 10

measurements. For QLS experiments the lipid concentration used was 0.3 mg ml^{-1} or 0.44 mM l^{-1}.

The measured correlation functions of the scattered intensities were analysed using a second order cumulant development which allowed the determination of the average translational diffusion coefficient D and the polydispersity factor. D is related to the hydrodynamic radius R_h via the Stokes-Einstein relation, $R_h = k_B T/6\pi\,\eta\,D$ where η is the viscosity of the solvent at the temperature T.

FT-IR spectroscopy

Infrared spectra were obtained using a Perkin Elmer 1760 Fourier transform spectrophotometer. Each spectrum is an average of several scans with a spectral resolution of 1 cm^{-1}. DMPC unilamellar bilayers were prepared in 150 mM NaCl, 10 mM Tris—HCl, pH 8.3, H_2O or D_2O buffers depending on the spectral region investigated. The lipid concentration used was typically 30 mg ml^{-1}. The spectra were recorded at discrete temperatures with a waiting time of 15 min between two subsequent spectra.

Titration of propranolol and partition coefficient

Propranolol in aqueous solutions shows two UV absorption bands at 260 and 215 nm. The intensity of these bands can be used to titrate the drug and to check its partition between the lipid membrane and the aqueous external medium. For this purpose, after the addition of drug, the vesicle solutions were dialysed at constant temperature through Spectrapore membranes (cut-off MW 3500). After 24 h the concentration of the drug in the dialysate was measured. The partition coefficient K_p defined as K_p = [conc (g/g) of propranolol in lipid phase]/ [conc (g/g) of propranolol in water phase] was evaluated knowing the drug concentration in the dialysate, the volume V_o of the dialysed dispersion and the volume V of the buffer used for the dialysis.

Results

Temperature dependence of LUV size

It is well known that phospholipidic vesicles undergo phase transitions when the temperature is changed. For DMPC vesicles the transition point has been measured by different physical methods (DSC, ESR, IR, Raman spectroscopy, QLS, etc. [26—31]) and was found at 24°C. This transition point separates two phases, a crystalline liquid phase above the transition point and a gel phase below this temperature. The physical properties of membranes vary from one state to the other: the molecular arrangement of the hydrocarbon chain is modified. As a consequence, the area per head group changes too and thus the total area of the vesicles is altered depending on the temperature, as shown in Fig. 1a. By measuring the hydrodynamic radius R_h by QLS, one can monitor this phase transition. By cooling, the plot shows a decrease of the size with the midpoint at 24°C which is the transition point of the pure lipid vesicles.

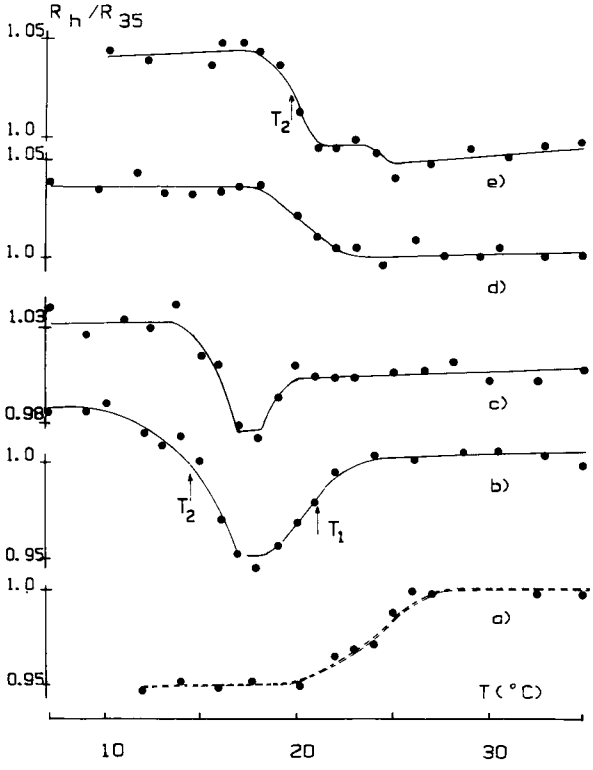

Fig. 1. Temperature dependence of the hydrodynamic radius R_h of DMPC LUV in the presence of D-propranolol at different concentrations. The ordinate represents R_h normalized to the value R_{35} of pure lipid vesicles at 35°C. The vesicles are in buffers containing 150 mM NaCl, 10 mM Tris—HCl, pH 8.3 and D-propranolol of concentration (a) 0 mM (b) 0.12 mM, (c) 0.22 mM, (d) 0.33 mM, (e) 0.44 mM. Lipid concentration: 0.44 mM.

When the drug is present, the knowledge of the thermal behavior of the bilayer is very useful for understanding the drug-bilayer interaction. Figures 1b—1e show the variation versus temperature of the DMPC LUV size in the presence of different concentrations of D-propranolol. The plots represent the vesicle hydrodynamic radius R_h normalized to the value R_{35} of pure lipid vesicles at 35°C. It is noticeable that the change in vesicle size does not exceed 1% when D-propranolol is added at 35°C. In the presence of propranolol, the plots show two abrupt changes corresponding to two steps of the transition. When the vesicles undergo the first step of the transition, a decrease of the vesicle size is observed at small drug concentration. The transition point T_1 is shifted towards a temperature lower than that of the pure lipid vesicles and varies as a function of the drug concentration as indicated in Fig. 2. At the same time, when the propranolol concentration c is increased, the decrease of the vesicle size is less and less pronounced in such a manner that at about c = 0.33 mM l^{-1}, it seems that T_1 disappears as it is shown on Figs. 1d—e.

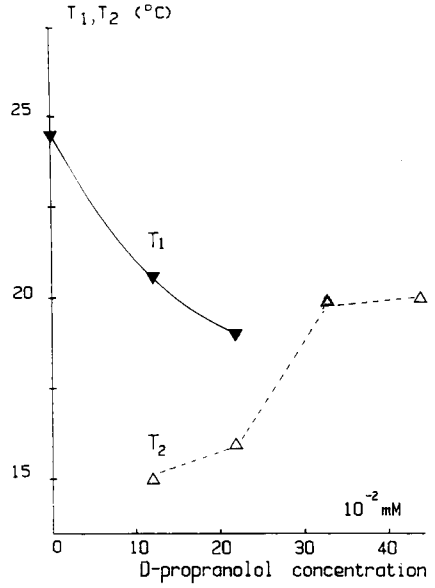

Fig. 2. Variation of the transition points T_1 and T_2 of DMPC LUV against D-propranolol concentration. T_1 and T_2 were determined from the midpoints of the plots in Fig. 1.

The second step of the transition occurs at a temperature T_2 lower than T_1. When the vesicles undergo this transition the vesicle size is increased and this increase becomes more and more important when the drug concentration is increased. With c= 0.44 mM l^{-1}, the value of R_h at low temperature is 104.5% of the value obtained at 35°C. At this temperature the vesicle size is larger than in the liquid-crystalline phase. Concerning the transition point T_2, it shows an abrupt change at c=0.22 mM l^{-1} (Fig. 2). It is notable that this increase has not been observed in small vesicles.

Infrared spectroscopy

Fourier transform infrared spectroscopy was used in order to investigate the effect of D-propranolol on vesicles at a molecular level. Two absorption spectral regions of major interest are affected by the presence of the drug, corresponding to the stretching vibration modes of the CH_2 groups and the PO_2^- groups.

(a) Effect of propranolol on the spectral region on 2800—3000 cm^{-1}. The wavenumbers of the stretching vibrations of the methylene groups in the spectral region from 2800 to 3000 cm^{-1} are very sensitive to conformational changes in the hydrocarbon chains. In particular, the strong absorption band at 2850 cm^{-1} assigned to the symmetric stretching vibration of the methylene groups, shows temperature-induced shifts reflecting the change of the *trans/gauche* ratio of the acyl chains conformers. Temperature variation induces a shift of the CH_2 stretching band as it is shown in Fig. 3. Wavenumbers near 2852 cm^{-1} are characteristic of conformationally disordered polymethylene chains with a high content of *gauche* conformers (generally observed in the liquid-crystalline state) while lower values are characteristic of ordered methylene chains as found in the gel state [29]. For pure DMPC vesicles (Fig. 3a), the transition point near 24°C separates two states, in agreement with the shift of this band. When D-propranolol is added into the vesicle solution, the feature is completely changed: the liquid crystalline-gel phase transition is very broadened. In the liquid-crystalline phase, the ad-

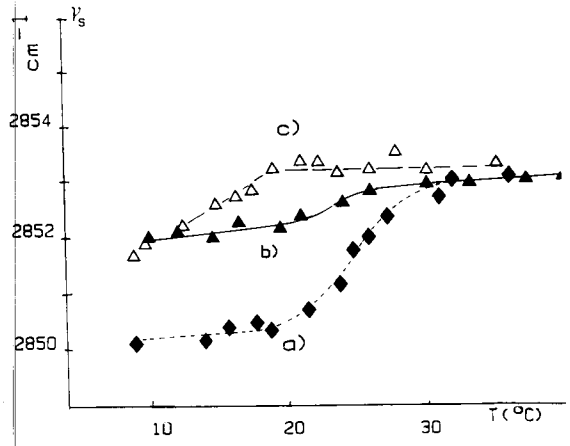

Fig. 3. Temperature dependence of the wavenumber of the symmetric CH_2 stretching mode of DMPC LUV in the absence (a) and in the presence (b and c) of propranolol at pH 8.3 (b) and pH 5.4 (c). DMPC/drug = 2/1 mol/mol.

dition of propranolol does not cause any effect in the membrane. On the other hand, below the transition point, the propranolol has an effect of disordering the acyl chains in the gel phase. We can even say that at high concentrations, the transition disappears.

(b) Effect of propranolol on the spectral region 950—1350 cm^{-1}.

Figure 4 shows infrared absorption bands in the absence and in the presence of D-propranolol, respectively. In the absence of the drug (Fig. 4a) the spectrum is characterized by two strong bands at 1088 cm^{-1} and 1232 cm^{-1} corresponding to the symmetric and antisymmetric stretching modes of the PO_2^- groups of the polar heads. The band at 1088 cm^{-1} is shouldered by a band at 1068 cm^{-1} assigned to the coupling between the carbonyl groups and the adjacent C—C bond. In the presence of D-propranolol (Fig. 4b) the band at 1088 cm^{-1} is shifted towards 1094 cm^{-1} and the band at 1231 cm^{-1} shifted to 1224 cm^{-1}. Moreover, the relative height of the band at 1088 cm^{-1} with respect to the CO/CC band at 1068 cm^{-1} is modified. These effects are indicative of an interaction between the drug molecules and the PO_2 groups of the membranes. The absorption bands

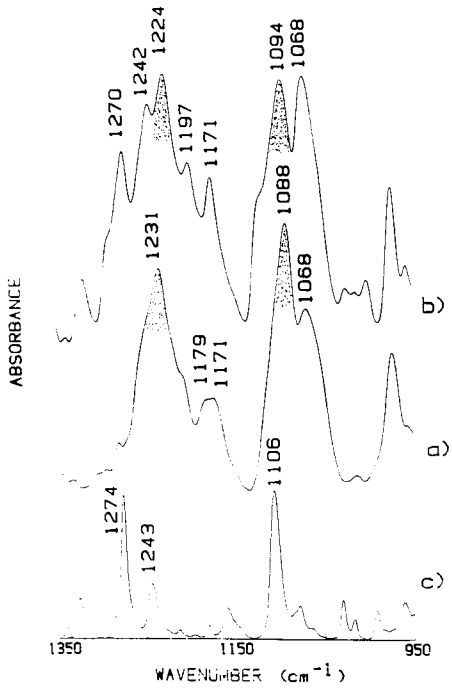

Fig. 4. FT-IR spectra of (a) pure DMPC LUV, (b) DMPC LUV in the presence of D-propranolol in the ratio drug/lipid = 1/2 mol/mol, (c) pure D-propranolol. Buffers H_2O, 10 mM Tris, 150 mM NaCl pH 8.3. The subtraction of pure propranolol absorption bands is difficult because of their frequency shifts.

of the drug detected at 1242 cm^{-1} and 1105 cm^{-1} do not interfere with these effects.

On the other hand, the band at 1179 cm^{-1}, due to the wagging of the CH_2 groups, is drastically altered in the presence of D-propranolol. This may be an indication that the drug disturbs the acyl chains.

The partition coefficient

With the concentration of propranolol found in the dialysate, we have deduced the amount of the drug unbound to the bilayer. In this calculation, we have taken into account the internal volume V_l of the vesicles evaluated from their size, R_h measured by QLS, the lipid density ρ (1.08 g cm^{-3} at 4°C or 1 g cm^{-3} at 35°C), the thickness e (50 Å at 4°C or 34 Å at 35°C) of the bilayer, and the lipid total mass m_l in the initial solution:

$$V_l = \frac{m_l R_h(1 - 3e/R_h)}{3e\rho}$$

the mass m_3 of the unbound drug is thus:

$$m_3 = \frac{C\,V_o(V + V_o - V_1)}{V_o - V_1}$$

Table I summarizes the results for the partition coefficient at different temperatures. At 4°C, the partition coefficient is higher than that at 35°C.

Discussion

From the above results, insights in the effect of D-propranolol on the physical state and on the thermal behavior of the DEC large unilamellar vesicles have been obtained. Measurements of the vesicle size by QLS reveal that the drug shifts the transition point and alters the low temperature phase of the model membranes. The study by FT-IR spectroscopy shows that drug molecules interact with the polar heads of the lipid molecules. These results allow a scheme to locate the drug molecules in the membrane from which their effect can be explained.

First, the plot 1a in Fig. 1 representing the variation of the vesicle size versus temperature shows unambiguously two states of the pure DMPC LUV. The difference of 5—6% in the vesicle size at the liquid crystalline to gel-like phase transition corresponds to a change of 10—12% in the molecular section area of lipid molecules. This value is in good agreement with that predicted [32] or observed by other workers [33—34]. For membranes in the presence of D-propranolol, the plots 1b—c clearly indicate three states separated by two transitions T_1 and T_2. In cooling from the liquid crystalline state, the vesicles undergo a first transition T_1 and have a tendency to reach a more ordered state as do vesicles in the absence of drug, with the transition point shifted towards lower temperatures. However, in the state just below T_1, the vesicle size is larger than that in the gel-like state, and this phase gradually looses the characteristics of a "usual" gel phase when the drug concentration is increased. The vesicle size reaches its value in the liquid crystalline phase at c= 0.33 mM and one can say that T_1 disappears. This first transition T_1 seems to be only a prearrangement of the lipid molecules before the second transition occurs at T_2.

Concerning T_2, it is strongly affected by the drug concentration. It may be that is related to the pretransition of DMPC vesicles occuring at 13—14°C. This pretransition is very weak and generally not observable by quasielastic light scattering for pure DMPC vesicles but it may be enhanced by the presence of the drug. A perturbation on the pretransition by guest molecules such as phenol or salicylic acid has been observed in DPPC vesicles [35].

When the vesicles undergo this second step, their size in the presence of the drug, R_h, becomes much larger than in the absence of the drug. The ratio of the size increase reaches 10%, which corresponds to an increase of the vesicle area of 20% and the vesicle area becomes even larger than that at high temperature in the liquid crystalline state. It is interesting to note that when D-propranolol solutions are added at 35°C, there is only an insignificant change in size of the vesicles. From dialysis data showing 4% and 15% of the whole drug molecules bound to the vesicles at 35°C and at 4°C (below T_2), respectively, the change in area simply due to the bound drug molecules below T_2, estimated as 15/4 times the change at 35°C, should also be small and cannot exceed 4%. In other words, an increase in area as important as 20% observed at low temperature would indicate a new situation.

In order to explain this, it is important to consider the structure of propranolol molecules (Fig. 5) and the results of FT-IR spectroscopy. The drug is amphiphilic, possessing an aromatic group at

TABLE I

The percentage of the mass bound to the LUV DMPC bilayer (in 10 mM Tris—HCl, 150 mM Nacl, pH 8.3 buffer) and the partition coefficients K_p, K_d of D-propranolol. Lipid concentration, 0.42 mM l^{-1}; drug concentration, 1 mM l^{-1}.

T (°C)	% bound to the bilayer	K_p (in weight fraction units)	K_d (in mole fraction units)
4	15	418	24.5
35	4	98	5.7

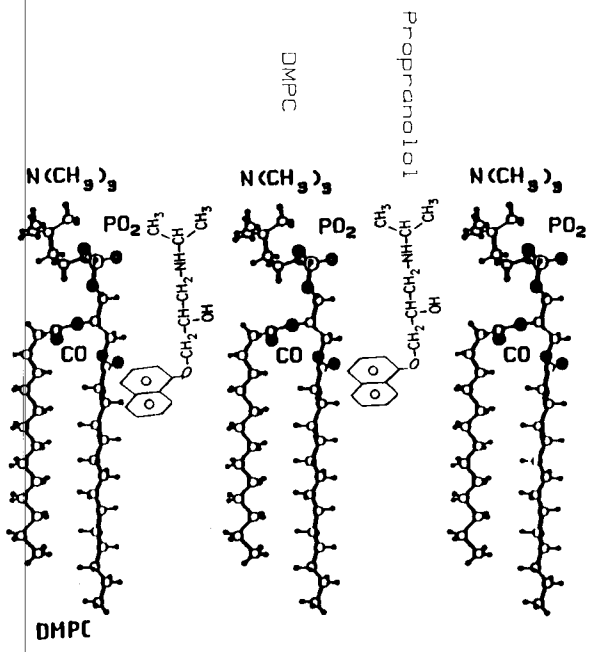

Fig. 5. Molecular structures of propranolol and DMPC and model proposed for the interaction of D-propranolol and DMPC LUV at low temperature. Vacant places are available for interdigitation and/or disordering of the acyl chains.

one side and a tertiary amine group at the other side, with a $pK_a = 9.45$. In the actual experimental conditions, at pH 8.3, the drug molecules are positively charged. The alteration of the absorption bands at 1088 cm^{-1} and at 1232 cm^{-1} assigned to the symmetric and antisymmetric stretching modes of PO_2^- unambiguously reveals an interaction between the drug and the phosphate groups. This interaction is undoubtedly coulombic in nature and occurs between the PO_2 group and the quaternary ammonium group of the propranolol molecule, taking into account the pH value lower than its pK_a. Such an interaction must be related to the increase of the zeta-potential observed by Schlieper et al. [21] when propranolol is added to phosphatidylcholine vesicles. This result allows us to locate the drug molecule at the aqueous interface of the bilayer. Moreover, the shift of the transition point and the change in the wavenumber of the CH_2 symmetric stretching mode at low temperatures prove that the hydrophobic aromatic group of the drug penetrates into the bilayer core. However, the molecule is not sufficiently long, even in its full extended conformation, to penetrate deeply into the membrane as schematized in Fig. 5.

From these observations, it is plausible that the presence of D-propranolol molecules leaves vacant places in the hydrophobic region of the lipid bilayer and to accommodate the new situation, there are possibilities for either the disordering of the neighbouring acyl chains or for the penetration of the acyl chains of the other monolayer. We will consider both possibilities.

Concerning the disordering of the neighbouring acyl chains, it can be indicated by the modification of the wavenumber $\nu(CH_2)$ of the symmetric stretching mode of the methylene groups. At low temperatures the wavenumber $\nu(CH_2)$ is higher in the presence than in the absence of the drug as shown in Fig. 3, and the effect is more pronounced at low pH (Fig. 3c). This may indicate a more disordered state of the acyl chains than in the gel state of pure lipid vesicles. Such a disordering has also been observed in the case of DPPC vesicles in the presence of tetracaine [15]. At low pH, the propranolol molecules are more protonated than at alkaline pH, the drug is more attached to the PO_2 group and penetrates less into the depth of the bilayer, favouring the disordering of the CH_2 chains.

However, the whole results are rather in favour of an interpenetration of the acyl chaines of the apposing monolayers for three reasons. First, although there is disordering shown by the high value of $\nu(CH_2)$ in the low temperature phase, it is not greater than that in the liquid crystal phase. Such a disordering, energetically unfavourable at low temperature, should not allow an average sectional cross area larger than that in the liquid crystalline phase as observed by quasielastic light scattering (Fig. 1). Second, the partition coefficient at 4°C indicates 15% of the drug amount bound to the bilayer. This amount is larger than that in the high temperature phase, implying an increase in the number of interaction sites or vacant places. Finally, as estimated by Simon et al., interdigitation corresponds to an important gain of Van der Waals energy [6]. Therefore, from the in-

crease in size observed by quasielastic light scattering and these considerations, we believe that we are dealing with an interdigitated state at low temperature. Taking into account the fact that in the presence of D-propranolol the area increases by only 20%, and the $\nu(CH_2)$ indicates a disordered state, we infer that the interdigitation is partial.

Conclusion

With two complementary techniques, quasielastic light scattering and Fourier transform infrared spectroscopy, we have shown the interaction of the cationic D-propranolol drug with DMPC vesicles. Whereas FT-IR Spectroscopy allowed us to locate the drug molecules at the aqueous interface of the bilayer, QLS revealed that with large size unilamellar vesicles, besides the shift of the transition point, the drug alters the gel phase of the membrane and induces a second transition to a phase which would be an interdigitated one. This last point might be related to the "stabilizing" effect of D-propranolol at high doses.

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