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Interaction of filipin with dimyristoylphosphatidylcholine membranes studied by ^{2}H-NMR, circular dichroism, electronic absorption and fluorescence

J. Milhaud [1], J. Mazerski [2], J. Bolard [1], and E. J. Dufourc [3]*

[1] Laboratoire de Physique et Chimie Biomoléculaire (UA CNRS 198), Université Pierre et Marie Curie, 4 Place Jussieu, F-75252 Paris Cedex 05, France
[2] Department of Pharmaceutical Technology, Technical University, PL-80952 Gdansk, Poland
[3] Centre de Recherche Paul Pascal, CNRS, Domaine Universitaire, F-33405 Talence Cedex, France

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Abstract. Interaction of the pentene antibiotic filipin with dimyristoylphosphatidylcholine (DMPC) membranes has been monitored by ^{2}H-NMR, circular dichroism (CD), electronic absorption and fluorescence in the temperature range 10° to 60 °C. Interaction appears to depend on whether filipin is added before or after membrane formation and also upon the temperature of the system.

When filipin is added to preformed DMPC large unilamellar vesicles (LUV), the association constants, as determined by electronic absorption are 39×10^3 M^{-1}, 15×10^3 M^{-1} and 0.6×10^3 M^{-1} at 15°, 30° and 50 °C, respectively. Under identical conditions, CD spectra of bound filipin exhibit features characteristic of an aggregation over the whole temperature range.

When filipin is incorporated in membranes during their preparation, the ^{2}H-NMR spectra of deuterated DMPC indicate that the drug has a slight disordering effect on the lipid matrix below the temperature, T_c, of the gel-to-fluid phase transition and above $T_c + 11$ °C. Between these two temperature boundaries the system consists of two lipid regions of very different dynamic properties. One of the regions, which is attributed to a filipin-lipid complex, has the properties of gel-like lipids whereas the other has those of fluid-like lipids. The latter domain is however more ordered than the pure lipid at corresponding temperatures. CD spectra under the same conditions are found to be identical to spectra when the drug is added to preformed membranes, only in the region T_c to $T_c + 11$ °C.

Filipin induced carboxyfluorescein release from DMPC-LUV is found to be complete when the filipin-to-lipid ratio is near 1, for temperatures below $T_c + 11$ °C.

Results are compared to previous data on amphotericin B and provide evidence that the gel-like structure of phospholipid and membrane permeation may be induced by filipin even in the absence of cholesterol.

Key words: ^{2}H-NMR, filipin, DMPC, circular dichroism, model membranes, carboxyfluorescein release

Introduction

Polyene antibiotics are known to modify the properties of natural and model membranes, especially their permeability (Bolard 1986).

The pentene antibiotic filipin is not used clinically because of its high toxicity. Nevertheless the filipin complex (a mixture of several pentenes, see Bergy and Eble, 1968) has been widely used as a probe for cholesterol in biological membranes, particularly since the demonstration that in membranes containing 3-β-hydroxysterols, filipin induces characteristic deformations or lesions as observed in freeze-fractured or negatively stained membranes (for reviews see Robinson and Karnovsky 1980; Bittman et al. 1983; Miller 1984; Behnke et al. 1984).

However some recent studies questioned the use of filipin as a probe for cholesterol in biological membranes (Miller 1984; Behnke et al. 1984): does the absence of lesions indicate the absence of cholesterol and does the number of filipin-induced lesions in a particular area of a membrane necessarily reflect the concentration of cholesterol in that area?

Deuterium solid state NMR (^{2}H-NMR) has recently been used to investigate, at the molecular level and by means of deuterium labeled lipids and sterols, the interactions of filipin with sterol-containing DMPC bilayers (Dufourc and Smith 1985). Filipin was reported to complex cholesterol very strongly. This antibiotic totally immobilizes sterol molecules in the DMPC membranes. This complex formation was found to be temperature dependent since at 0 °C, in

* To whom offprint requests should be sent

Abbreviations: NMR = nuclear magnetic resonance; CD = circular dichroism; DMPC = dimyristoylphosphatidylcholine; EPA = egg phosphatidic acid; LUV = large unilamelar vesicles; SPC = soybean phosphatidylcholine; DMSO = dimethylsulfoxide; CF = carboxyfluorescein

DMPC, all sterols were segregated whereas at 60 °C they were almost totally free from interaction with the antibiotic. On the other hand, the lipids of the same system were not showing any changes in ordering upon filipin addition except at high temperatures where they exhibit lower orientational order in the presence of the antibiotic.

On the other hand, circular dichroism may be used to follow conformational changes and self-association of polyene antibiotics (Mazerski et al. 1982). It was recently shown by this method that, in soybean phosphatidylcholine (SPC) large unilamellar vesicles (LUV), filipin was interacting with phospholipids (Milhaud et al. 1988).

We report herein upon the effects of filipin on DMPC model membranes using ^{2}H-NMR, electronic absorption, fluorescence and circular dichroism. Results confirm the "screen" model, i.e. the proposal that sterols prevent antibiotics from direct interaction with lipids (Dufourc et al. 1984a, 1986; Dufourc 1988), and clarify previous data on filipin-cholesterol-DMPC systems, thus providing a more complete description of the mode of action of this antibiotic on membranes.

Materials and methods

Materials

[*sn*-2-$^2H_{27}$] DMPC was prepared by acylation of 1-myristoyl-*sn*-glycerol-3-phosphocholine with [$^2H_{27}$] myristic acid according to Perly et al. (1984). Deuterated myristic acid was purchased from Larodan Lipids (Sweden) and Lyso DMPC prepared from egg yolks. Filipin and unlabeled DMPC were obtained from Sigma (La Verpilliere, France).

To facilitate the comparison of our results with those already published we have used the crude unfractionated filipin complex, as it is generally used in probe studies. The four main compounds of this complex (Bergy and Eble 1968) are undistinguishable by circular dichroism (CD). Concentrations of filipin solutions were determined by UV absorption ($\varepsilon_{355} = 8.7 \times 10^4 M^{-1}$ cm^{-1} in methanol). 5 [6] carboxy fluorescein was supplied by Eastman-Kodak and purified according to Ralston et al. (1981). Deuterium-depleted water (Aldrich, Strasbourg, France) was used to prepare NMR samples.

Methods

NMR spectroscopy: Model membranes were prepared as follows. Filipin and DMPC were dissolved, in appropriate amounts, in a chloroform: methanol (2 : 1) mixture (Dufourc and Smith 1985). The solvent was removed by a stream of nitrogen gas and further evaporation performed under vacuum at low temperature. The residue was then dispersed in deuterium-depleted water and lyophilized overnight at room temperature to obtain a fluffy powder which was subsequently dispersed in the same medium (lipid concentration of 50 mM). Samples were then vortexed and freeze-thawed several times to ensure homogeneity. During preparation and NMR experiments samples were kept in the dark. ^{2}H-NMR spectroscopy was performed on a Bruker MSL-200 spectrometer operating at 30.7 MHz. NMR signals were obtained by means of the quadrupolar echo sequence (Davis et al. 1976) with proper phase alternation of both transmitter and receiver. Quadrature detection was used and temperature regulated to ± 1 °C. Samples were allowed to equilibrate at a given temperature for at least 30 min prior to recording the NMR signal. Data treatment was performed on a VAX/VMS 8600 computer.

Optical techniques: The binding of filipin was studied according to two protocols: Filipin was added to preformed DMPC vesicles or incorporated into vesicles during their preparation. Large unilamellar vesicles were prepared by the reverse-phase evaporation procedure (Szoka and Papahadjopoulos 1977). Preparation was carried out entirely at 35 °C by adding 3 ml of chloroform to 3 ml of diethyl ether for 1 ml of aqueous phase (100 mM Na_2SO_4 buffered with 3 mM Na_2HPO_4 at pH 7.6). A small percentage (4% to 10% PL molar ratio) of egg phosphatidic acid (EPA) was always incorporated to prevent aggregation and sedimentation of DMPC vesicles. A stock filipin solution ($10^{-2} M$) in DMSO was made daily and appropriate quantities of this solution were dissolved in the buffer to obtain the desired lipid-to-antibiotic molar ratio (R_i): the final DMSO percentage was always lower than 2%.

For incorporation of filipin during LUV preparation, a $4.10^{-3} M$ filipin buffered solution was directly added with organic solvents to 10 µmoles of phospholipids before sonication and reverse phase evaporation: mixed DMPC/EPA/Fil LUV (66 : 4 : 30 in molar proportion) resulted from this procedure.

UV absorption measurements were carried out on a Cary 219 spectrophotometer and CD spectra were recorded on a Jobin Yvon Mark IV dichrograph. All spectra were recorded after 1 h sample equilibration at indicated temperatures. Each absorption spectrum was corrected for light scattering by vesicles. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (mol^{-1} cm^{-1}).

Fluorimetric measurements of carboxyfluorescein (CF) release were performed as follows. A 0.1 M solution of CF was incorporated into the LUV and the non-trapped dye was removed by gel filtration through Sephadex G 25M columns (Pharmacia). At

this concentration the dye fluorescence was self-quenched (Weinstein et al. 1977). After 15-min incubation of the marked LUV with filipin, a dye leakage depending on the filipin concentration occurred. The released dye was diluted in the external medium and its fluorescence was observed with an emission maximum at 519 nm with excitation at 490 nm. For each filipin concentration, the CF fluorescence increase, after filipin treatment, was normalized to the fluorescence increase corresponding to the complete release of sequestered CF induced by the addition of 0.05% (v/v) triton X-100, which is known to induce total disruption of LUV.

Results

Binding of filipin to preformed LUV

Electronic absorption. Electronic absorption has been widely used to monitor the binding of filipin to sterols or to lipid vesicles (Norman et al. 1976). In the present study, in the absence of sterol, we have used the method of Bittman et al. (1974). According to this method, the decrease of the absorbance values (A) at 358 nm is a measure of the amount of bound filipin. The concentrations of bound filipin (C_B) in the presence of vesicles were calculated using the expression:

$$C_B = \frac{\varepsilon_F C_T - A/l}{\varepsilon_F - \varepsilon_B},$$

where ε_F ($6.02 \times 10^4 M^{-1}$ cm^{-1}) is the extinction coefficient of free filipin, C_T is the total concentration of filipin, l is the light path length and ε_B (4.77×10^4 M^{-1} cm^{-1}) is the extinction coefficient of the bound form of filipin at 398 nm. We have studied the filipin DMPC LUV binding at 15°, 30° and 50 °C. Results are given in Fig. 1.

From NMR results (see below) it seems reasonable to assume a 1:1 stoichiometry for the phospholipid (PL)-filipin association. The corresponding association constants resulting from linear plots of the PL-bound/PL-free ratios as a functions of PL-bound are the following: $K_a = 39 \times 10^3 M^{-1}$ at 15 °C, $K_a = 15 \times 10^3 M^{-1}$ at 30 °C and $K_a = 0.6 \times 10^3 M^{-1}$ at 50 °C, showing that the interactions are substantially weaker at elevated temperatures.

Circular dichroism. Filipin spectra in buffer were shown to be concentration dependent: at low concentration ([Fil] $= 2 \times 10^{-5} M$) three small negative peaks at 323, 338 and 356 nm ($\Delta\varepsilon \sim -2$) and a small broad positive peak at 243 nm ($\Delta\varepsilon = +2.5$) were observed. When the filipin concentration was increased up to values greater than or equal to $4 \times 10^{-5} M$ an exciton doublet appeared abruptly, centered at 295 nm

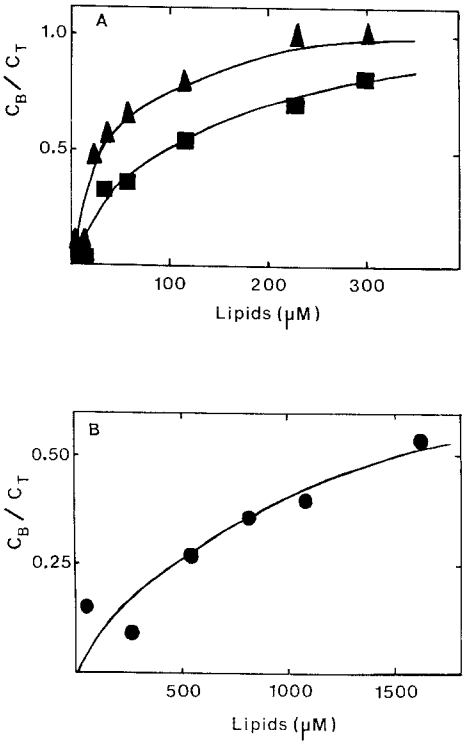

Fig. 1. Filipin association with dimyristoyl PC LUV monitored by electronic absorption at 15°, 30° and 50 °C. The plot shows the ratio of bound filipin (C_B) over total filipin (C_T) as a function of phospholipid concentration. C_B was determined from the absorbance at 358 nm (see text). *Part A:* $C_T = 10^{-5} M$; (▲ 15 °C, ■ 30 °C). *Part B:* $C_T = 2.2 \times 10^{-5} M$; ● 50 °C

($\Delta\varepsilon_{302} = +13$ and $\Delta\varepsilon_{289} = -16$ for [Fil] $= 12 \times 10^{-5} M$) as already described (Lemâtre and Moulki 1976). This spectrum intensifies with filipin concentration (Fig. 2).

In the presence of sterol-free DMPC LUV new spectra appeared (Fig. 2). In the lipid gel state (15 °C), in the presence of increasing LUV concentrations the spectrum of free filipin was progressively replaced by a new one for $0.08 \leq R_i \leq 4$. This spectrum was observed for filipin concentrations as low as $10^{-5} M$. It was characterized, for $R_i \leq 0.7$, by three positive peaks at 365 ± 3, 346 ± 3 and 330 ± 3 nm and a negative peak at 300 ± 5 nm.

In the lipid fluid state (30 °C), a similar spectrum was observed.

Binding of filipin incorporated during membrane preparation

^{2}H-NMR Spectroscopy. Figure 3 shows deuterium NMR powder spectra of [*sn*-2-$^2H_{27}$]-DMPC dispersions, in the gel phase at 10 °C (the gel-to-fluid phase transition temperature, T_c, of DMPC dispersions is 24 °C and that of [*sn*-2-$^2H_{27}$]-DMPC is 22 °C), in the

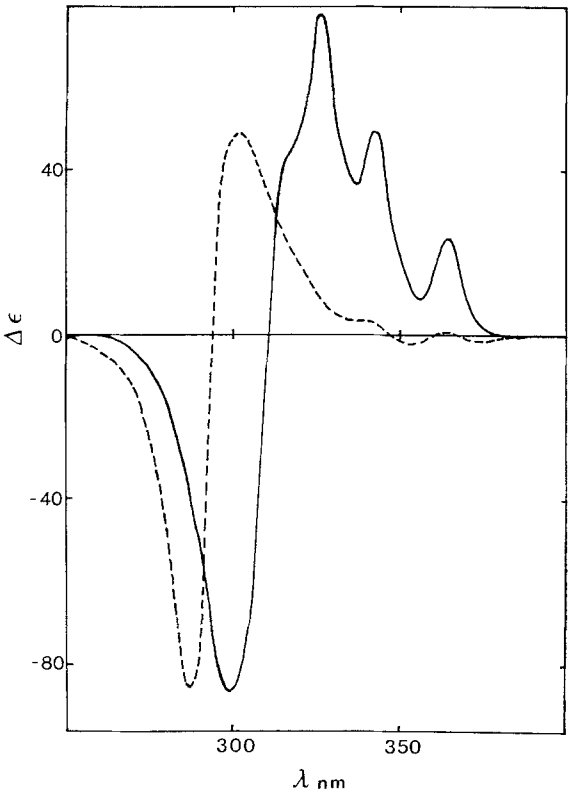

Fig. 2. CD spectra of filipin. *Broken line:* 16×10^{-5} *M* in buffer (100 m*M* Na_2SO_4, 2 m*M* Na_2HPO_4, pH = 7.6); *Full line:* 12×10^{-5} *M* in the presence of DMPC LUV at 15 °C PL concentration: 5×10^{-5} *M* ($R_i = 0.4$)

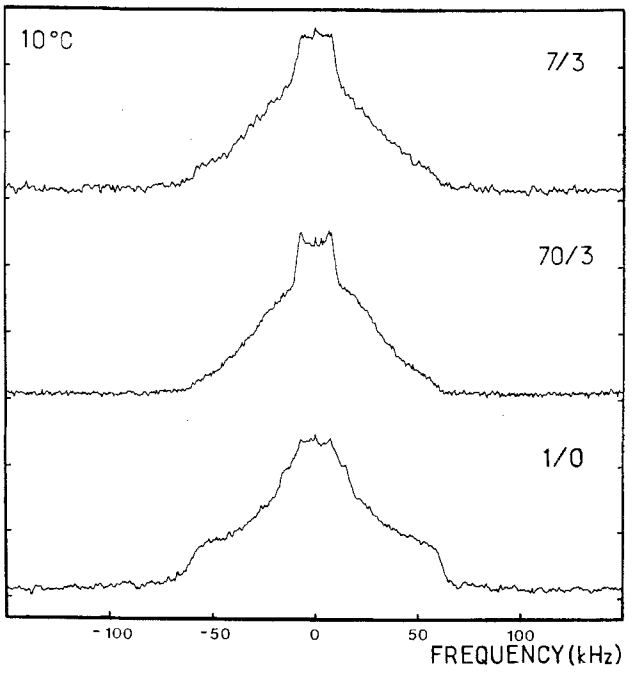

Fig. 3. ^{2}H-NMR powder spectra of [*sn*-2-$^2H_{27}$]-DMPC dispersions in the absence or presence of filipin, at 10 °C. Lipid-antibiotic molar ratios are indicated in spectra. Experimental parameters: $\pi/2$ pulse length 4 μs; recycle time, 1.5–2 s; spectral window 500 kHz; 2000 accumulations; quadrature detection

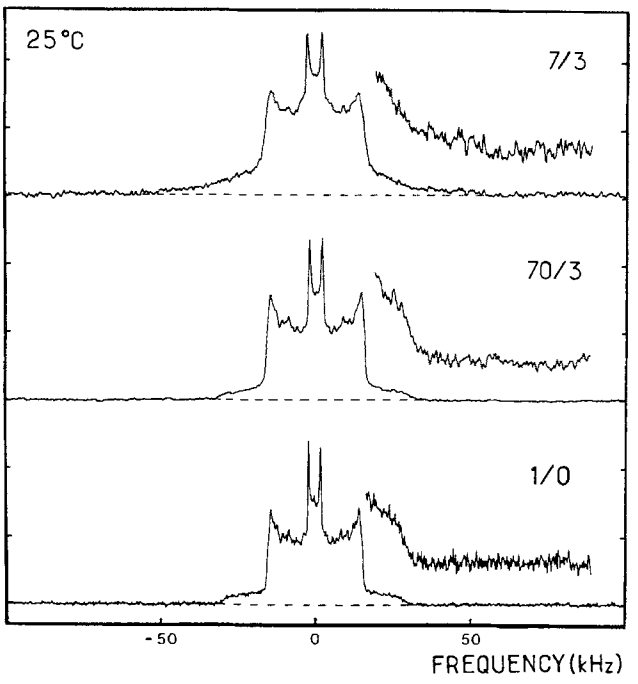

Fig. 4. As in Fig. 3 except T = 25 °C

presence or absence of filipin. The large powder pattern observed for pure DMPC indicates that the methylene segments of the *sn*-2 chain undergo very little motional averaging, the chain, being almost in an *all-trans* configuration (Davis 1983). It is clearly seen from this figure that the presence of antibiotic, even in very low doses (i.e., for $R_i = 70:3$), induces a narrowing of the deuterium powder pattern. Figure 4 is the equivalent of Fig. 3, except that the temperature of the system has been raised to just above T_c. In contrast to Fig. 3, one observes narrower and more structured spectra. In the case of pure DMPC, the observed powder pattern is characteristic of methylene segments undergoing fast axial reorientation to reduce the static quadrupolar interaction experienced by the deuterium nucleus. From the residual quadrupolar splitting which can be measured on the Pake doublets corresponding to each labeled position, the orientational ordering of each methylene segment can be obtained (Davis 1983; Seelig 1977). Upon addition of the antibiotic, this ordering is modified (see below). Moreover, for high amounts of antibiotic ($R_i = 7:3$), one detects the presence of a broad spectral component superimposed on a pattern similar to that of pure DMPC (Fig. 4, top). This new spectral component has a maximum width of ca. 120 kHz which corresponds to the spectral features of lipids in their gel phase. The area of this broad component has been determined as already described (Dufourc et al. 1984a). For $R_i = 7:3$, this component represents 26%, 18% and 7% of the total spectral area at 23°, 25° and 30 °C. Accuracy in

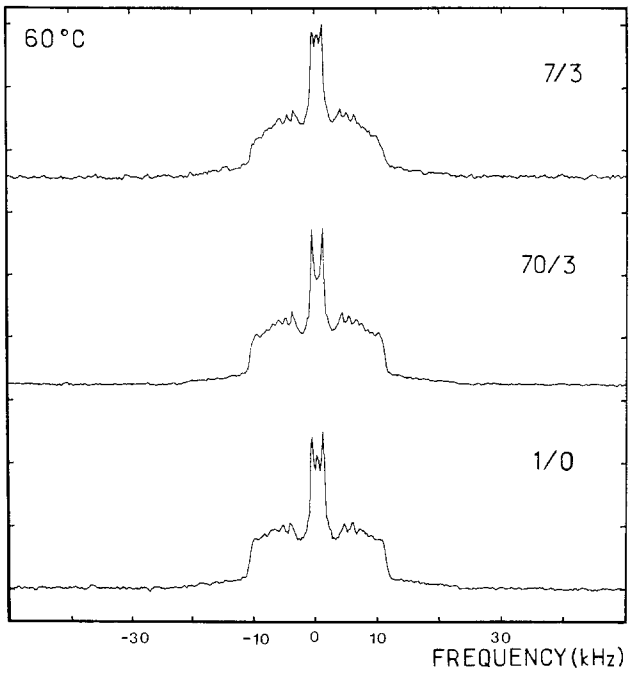

Fig. 5. As in Fig. 3 except T = 60 °C

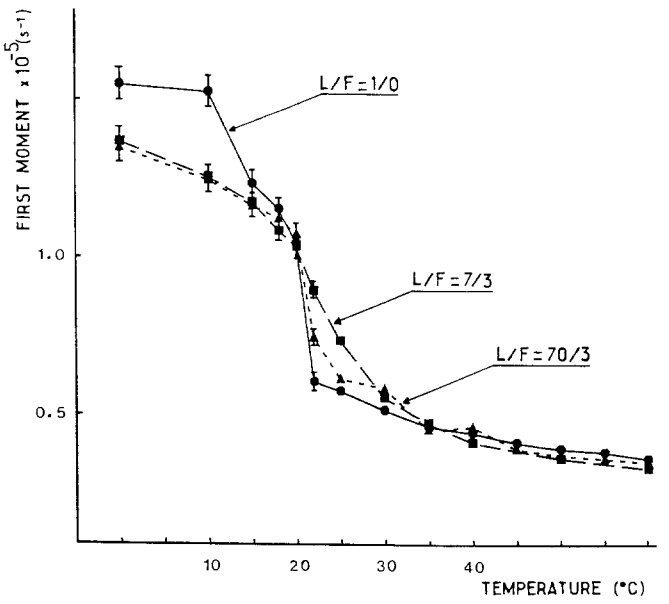

Fig. 6. Temperature dependence of first moments, M_1, of spectra of [*sn*-2-$^2H_{27}$]-DMPC in the presence or absence of filipin. Lipid-to-filipin molar ratios are indicated on each curve. *Bars* and *symbols* give an estimate of the error

area determination is ca. 5%. The broad spectral component is no longer detected on increasing the temperature to $T \gg T_c$ (Fig. 5). In addition, one notices in this figure that the more filipin added, the narrower the overall spectrum.

These results can be quantified by calculating the spectral moments (Davis 1979). When only one lipid phase is present, the first moment M_1 of [*sn*-2-$^2H_{27}$]-DMPC dispersions, gives an estimate of the overall ordering, integrated along the acyl chain length. For instance, Fig. 6 shows that the order–disorder transition of pure DMPC membranes is characterized by a sudden decrease of the first moment at T_c.

Addition of filipin modifies the thermal variation of M_1 : *i*) below T_c, the first moment decreases in the presence of filipin, *ii*) for $T_c \leq T < 35$ °C M_1 increases as the amount of drug in the system increases and, *iii*) at $T > 35$ °C M_1 slightly decreases when the antibiotic is added.

In the fluid phase, spectra can be "de-Pake-ed", i.e., one can perform a spectral deconvolution to get a sample-oriented-like spectrum from a powder spectrum (Bloom et al. 1981; Sternin et al. 1983). The residual quadrupolar splitting, and hence the orientational order parameter, can thus be very easily measured. Deconvolution has been performed for all spectra in the fluid phase. Assigment of quadrupolar splittings to labeled positions can be made by comparison with specifically labeled samples. The central doublet is thus straightforwardly assigned to the methyl terminal of the *sn*-2 chain. The variation of this splitting as a function of temperature and filipin content in the system is reported in Fig. 7. Filipin promotes different effects depending on the temperature. For $T_c \leq T < 35$ °C, the quadrupolar splitting increases as the amount of filipin increases. Above 35 °C, almost no change is observed for small quantities of antibiotic

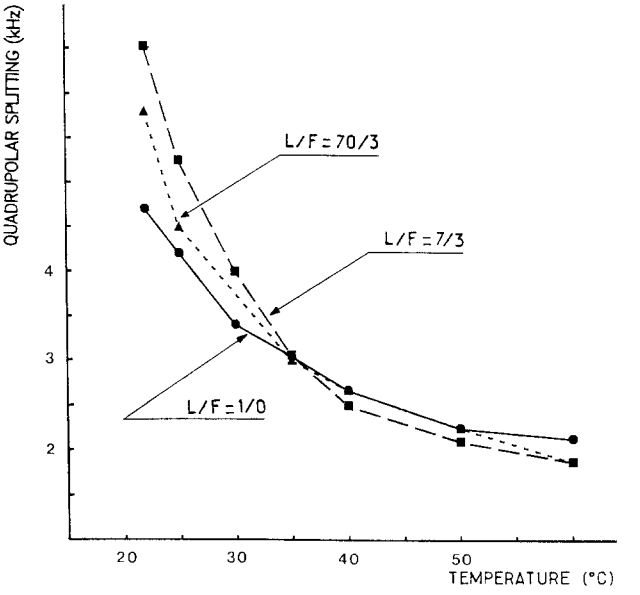

Fig. 7. Temperature dependence of the quadrupolar splitting of [14′-$^2H_3$]-DMPC dispersions in the presence or absence of filipin. Lipid-to-filipin molar ratios are indicated on each curve. Symbols give an estimate of the error

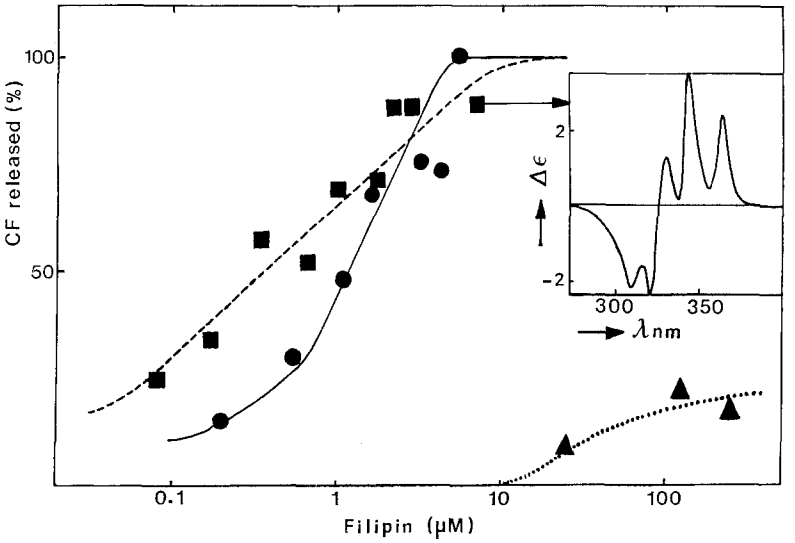

Fig. 8. Carboxyfluorescein release from DMPC/EPA (90:10) LUV as a function of the filipin concentration. *Broken line with black squares:* $[PL] = 5 \times 10^{-6} M$, 15 °C; *line with black circles:* $[PL] = 3 \times 10^{-6} M$, 30 °C; *dotted line with black triangles:* $[PL] = 5 \times 10^{-6} M$, 50 °C. *Inset:* CD spectrum of filipin in the presence of CF-containing DMPC/EPA LUV at 15 °C [PL] = $5 \times 10^{-6} M$, [Fil] = $1 \times 10^{-5} M$)

whereas a decrease in quadrupolar splitting is detected for higher amounts of filipin. The variation monitored for the methyl terminal is also observed for other labeled positions along the chain (not shown), i.e. the effects are perceived on the entire bilayer thickness.

Circular dichroism. Between T_c and $T_c + 11$ °C, the filipin CD spectra are similar in shape but relatively weak compared with those obtained by addition of the drug to preformed LUV. In contrast, below T_c new spectra are observed (data not shown). On increasing the temperature above $T_c + 11$ °C (40 °C) the spectrum became weak and largely featureless. These temperature-dependent spectral changes are reversible and instantaneous. Below T_c, weak, complex spectra are observed.

Filipin-induced carboxyfluorescein release

We studied the release of CF from DMPC/EPA (90 : 10) LUV by incubation with filipin at 15°, 30° and 50 °C (for 1 h at each temperature). The proportions of released dye are plotted on a semi-logarithmic scale as a function of filipin concentration (Fig. 8). At 30 °C the dose-response curve is relatively steep whereas at 15 °C the curve is flatter; both curves do, however, reach 100% release. In contrast, at 50 °C, the CF release is negligible. This may be related to the weak binding of filipin to DMPC LUV at this temperature (see electronic absorption).

Discussion

Interaction of filipin with model membranes consisting of DMPC, that is of saturated fatty acyl chains phospholipids, appears to depend on the mode of incorporation of the drug and on the temperature.

The binding of filipin to preformed DMPC LUV decreases with increasing temperature (i.e. $K_a = 39 \times 10^3 M^{-1}$, $15 \times 10^3 M^{-1}$, $0.6 \times 10^3 M^{-1}$ at 15°, 30° and 50 °C, respectively). Bittman et al. (1974) found $K_a \simeq 7 \times 10^3 M^{-1}$ (dissociation constant, K_d of $144 \times 10^{-6} M$) for filipin binding to lecithin membranes at 25 °C (i.e. in the fluid phase) which compares well with $K_a \simeq 15 \times 10^3 M^{-1}$ that we found for filipin binding to DMPC membranes at 30 °C. The CD spectrum of free filipin is modified by the presence of DMPC LUV. Over the whole temperature range this spectrum keeps the same doublet shape but its intensity decreases with increasing temperature, in agreement with the lowering of the affinity constant, observed by electronic absorption. This dichroic doublet, is red shifted but keeps the same sign as that of self-associated filipin in water. This indicates that filipin molecules keep the same spatial relationship between them upon binding. It is interesting to note that this bound species is observed at concentrations where free filipin in water is in the monomeric form. In other words binding of filipin to DMPC membranes appears to promote its aggregation.

When filipin is incorporated into membranes during their formation the distribution of the drug is supposed to be different from the previous case since it can reach both sides of the membrane. ^{2}H-NMR and circular dichroism studies were performed in similar conditions and their results can therefore be compared. The small amount of EPA present in the LUV used for the optical studies, is thought not to perturb the data, no detectable effect was observed on T_c.

a) ^{2}H-NMR studies

For temperatures lower than T_c or higher than $T_c +$ 11 °C the drug is seen to narrow the deuterium powder

patterns or reduce the quadrupolar splittings. This reflects a disordering of the lipid matrix, as induced by filipin.

For temperatures between T_c and T_c+11 °C and for $R_i=7:3$, a two component system is detected by NMR. These two regions of the lipid bilayer are in slow exchange ($>10^{-5}$ s) since they are distinguishable by NMR. One of these possesses the spectral features of a gel phase whereas the other has those of a fluid phase. This indicates that the motions of some of the lipids are much more restricted than the bulk. We believe that this broad component reflects a filipin-lipid complex. Moreover the remaining lipids are more ordered that the pure lipids, for the same temperatures. For $T_c \leq T \leq T_c+11$ °C the percentage of lipids contributing to the broad spectral component decreases on increasing the temperature. Since the filipin content is 30%, the lipid-to-antibiotic ratio in the complex can be estimated to be 1:1, 1:2, 1:4, at 23°, 25° and 30 °C, respectively. On increasing the temperature above 35 °C the broad component, as detected by NMR, disappears, that is, in the time scale of ^{2}H-NMR (10^{-5} s), the lipids involved in the complex dissociate on increasing the temperature. Above 35 °C, the antibiotic molecules would freely diffuse in the membrane plane which would result in the observed disordering effect. For lower doses of filipin ($R_i=70:3$), the behaviour is almost the same. The differences observed with higher doses can be explained by the smaller amount of lipid bound to filipin and therefore the higher proportion of free lipids. Disordering below T_c, ordering between T_c and T_c+11 °C and small disordering above 35 °C are observed. However, the broad spectral component is not detected. This is not very surprising since its area would be ca. 4%, assuming a lipid-to-drug ratio of 1:1 in the complex. This area is within the experimental error. Interestingly, the induced disordering effect below T_c is identical for $R_i=7:3$ and 70:3, whereas both the increased ordering between T_c and T_c+11 °C and the disordering promoted by the drug above 35 °C are dose dependent, i.e. the more filipin the more pronounced the effect (Fig. 6). The dose-independent effect in the gel phase can be understood as an impurity (filipin-lipid aggregate) effect on a solid-like matrix.

b) Circular dichroism

The spectra below T_c and between T_c and T_c+11 °C are different (not shown). Therefore, the differences in lipid dynamics as evidenced by ^{2}H-NMR in these two temperature domains, correlate well with differences in filipin spectral properties. When filipin was added to preformed LUV we did not observe these variations around T_c.

It is interesting at this stage of the discussion to compare the NMR and CD data with similar data obtained with another polyene antibiotic amphotericin B, for the same experimental conditions (Dufourc et al. 1984b; Milhaud et al. 1988). As with filipin, a complex involving gel-like lipids was observed upon amphotericin B addition. The apparent lipid-to-drug ratio was found to be 1:1 and was invariant with temperature, at variance to what is observed with filipin. This suggests that the association of DMPC with amphotericin B is stronger than with filipin.

The release of carboxyfluorescein from DMPC LUV observed in the presence of filipin confirms the interaction demonstrated by NMR and CD. The filipin-phospholipid association seems to be responsible for permeability inducement. This is shown in the inset of Fig. 8 where the filipin CD spectrum of CF encapsulated in DMPC-LUV treated with the amount of antibiotic that leads to 100% CF release appears to be very similar to that of filipin associated with cholesterol-free DMPC-LUV (cf. Fig. 2). Comparison of relative rates for filipin induced CF release in the presence and absence of cholesterol is under study. Nonetheless, it appears from the above that the presence of sterol in membranes is not a prerequisite to the inducement of permeability.

In earlier studies on cholesterol-containing membranes it was shown that filipin preferentially complexes cholesterol (Dufourc and Smith 1985). By analogy with similar studies on amphotericin B-cholesterol-lipid systems (Dufourc et al. 1984a), it was proposed that membrane sterols could surround the antibiotics in the complexes and thus prevent the polyene from direct interaction with lipids. The fact that the broad lipid component observed (in the present work) in filipin-DMPC systems disappears when cholesterol is present (Dufourc and Smith 1985) reinforces the model.

From the results presented herein, it appears that filipin may also complex pure lipids. Although no data are available, up to now, on the relative association constants between filipin and membrane sterols, the use of this antibiotic selectively to probe cholesterol concentration in membranes must be considered questionable.

Conclusion

The present study indicates clearly, and by means of four physico-chemical techniques, that filipin interacts with sterol-free membranes consisting of saturated fatty acyl chain phospholipids. The interaction is stronger with lipids of low dynamics. This can be understood if one considers that gel-phase lipids may possess some of the rigid character of membrane sterols to which filipin is known to complex in a strong manner. It must be noted that even with sterol-free

membranes constituted from unsaturated fatty acyl chain phospholipids (soybean PC), an interaction was demonstrated although the conformation of the bound drug was different from that observed with DMPC membranes (Milhaud et al. 1988).

The strength of complexation to DMPC membranes decreases with increasing temperature. For high temperatures with respect to T_c, no complex is detected by NMR and the antibiotic is seen to disorder the entire lipid matrix.

Amphotericin B and filipin appear to form the same type of complex with sterol-free lipid membranes with the difference that the lipid-to-antibiotic association is stronger with amphotericin B than with filipin. This reinforces the "screening" role proposed for sterols to explain the disappearance of these phospholipid-antibiotic complexes when both drugs are added to cholesterol-containing membranes (Dufourc and Smith 1985; Dufourc et al. 1986; Dufourc 1988).

Finally our results question the origin of lesions observed by electron microscopy on membranes treated with filipin and the nature of the permeating species (filipin-lipid or filipin-sterol complexes).

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