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Competition between cholesterol and phospholipids for binding to filipin

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The interaction of the polyene antibiotic filipin with large unilamellar vesicles (LUV) has been studied by circular dichroism (CD). (1) On the one hand, the interaction has been followed with preformed LUV. In the presence of cholesterol-free dimyristoylphosphatidylcholine (DMPC) LUV, monomeric filipin, above a critical concentration of about $1 \cdot 10^{-6}$ mol/l and up to a DMPC/filipin molar ratio of approximately 4, bound to LUV and self-associated in the membranes as demonstrated by the presence of an excitonic doublet. For increasing DMPC/filipin molar ratios, filipin remained bound to membranes but became more and more under monomeric form. In the presence of preformed cholesterol-containing DMPC LUV, for increasing concentrations of filipin, the formation of a 1:1 filipin-cholesterol complex was first observed; then, for an antibiotic/cholesterol molar ratio higher than 1 an interaction between filipin and phospholipid, identical to the one observed with cholesterol-free LUV, was observed. (2) On the other hand, the interaction with cholesterol-containing model membranes has been followed in the same experimental conditions as those used in recent studies by ^{2}H-NMR (Dufourc, E.J. and Smith, I.C.P. (1985) Biochemistry 24, 2420–2424) and by ESR (Maurin, L., Bancel, F., Morin, P. and Bienvenue, A. (1988) Biochim. Biophys. Acta 939, 102–110), that is with filipin incorporated into membranes during their preparation. For preparations with filipin/cholesterol molar ratios of about 1:1 we confirm the results obtained by both methods. In the presence of an excess of filipin (filipin/cholesterol molar ratio 10:1), the CD spectrum observed with egg-yolk PC at 6 °C (conditions used in the ESR study) is identical to that observed in the presence of pure DMPC above the transition temperature (Milhaud, J., Mazerski, J., Bolard, J. and Dufourc, E.J. (1989) Eur. Biophys. J. 17, 151–158). We conclude that in these conditions a filipin-phospholipid association occurs once the sterolic sites of fixation have been occupied.

Introduction

The polyene antibiotic filipin is toxic for animal cells and not used as a drug. In contrast it has been extensively used as a probe, to test the presence of cholesterol in membranes, since 1973; by then, several authors [1,2] showed that cholesterol-containing membranes, when they are treated with filipin, exhibit typical lesions (hemispherical bulges of 15–25 nm in diameter) easily observable on filipin-treated freeze-fracturated or negatively stained membrane [3–5].

Recent studies have, however, questioned the selectivity of filipin binding to cholesterol. First the affinity of filipin for cholesterol was found to be low when the affinity was calculated using molar fractions of reactants with respect to phospholipid [6]. Then, the interaction of filipin with different phosphatidylcholine (PC) was demonstrated. It occurs with egg-yolk PC small unilamellar vesicles (EPC SUV) [7], soybean PC large unilamellar vesicles (LUV) [8] as well as dimyristoyl-PC (DMPC) LUV [9]. Even if the affinity of filipin for the phospholipids is weaker than that for cholesterol it must be taken into consideration because the studies on filipin-treated freeze-fractured or negatively stained membranes are generally done in the presence of a large excess of filipin. In the present study we have first monitored by circular dichroism (CD) the interaction of

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Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; PC, phosphatidylcholine; EPC, egg-yolk phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; EPA, egg phosphatidic acid; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; DMSO, dimethylsulfoxide.

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filipin with cholesterol and phospholipids in preformed cholesterol-containing DMPC LUV taking advantage of the specific spectra corresponding to these two types of interaction [8]. Then, we have extended these CD studies to preparations obtained under the same experimental conditions as those used in recent studies by ^{2}H-NMR [9,10] with DMPC membranes or by ESR [6] with EPC membranes containing a paramagnetic analogue of cholesterol. We were then able to provide further information to interpret the results of ESR and ^{2}H-NMR. Indeed, CD has proved to be the most convenient method for monitoring conformational changes and self-association of polyene antibiotics [11,12].

Materials and Methods

Materials. EPC was prepared according to Ref. 13. Egg-yolk phosphatidic acid (EPA) and DMPC were purchased from Sigma and used without further purification. Cholesterol was purchased from Carlo Erba and was recrystallized in ethanol before use. Filipin was purchased from Sigma. In earlier studies using filipin as a probe for membranous sterols, this antibiotic was generally used in its crude unfractionated form; with the object of comparing our investigations to the previous ones, we thus proceeded in the same way. The four main compounds of this filipin complex [14] are undistinguishable by CD. All the solvents used were of spectroscopic quality.

Vesicles. The binding of filipin was studied according to two protocols: either filipin was added to preformed DMPC vesicles or it was incorporated into DMPC or EPC model membranes during their preparation. Preformed DMPC LUV were prepared by the reverse-phase evaporation procedure [15]. Preparation was entirely carried out at 35°C (above the transition temperature) and 3 ml of chloroform was added to 3 ml of diethyl ether for 1 ml of aqueous phase (10^{-1} mol/l Na_2SO_4 buffered with $2 \cdot 10^{-3}$ mol/l $Na_2H\,PO_4$ at pH 7.6). A small percentage (4–10% PL molar ratio) of EPA was always incorporated to DMPC vesicles to prevent their aggregation and their sedimentation. It was checked that this small amount of negative phospholipid had no effect on the CD spectra, by varying its percentage from 2 to 10 in the given type of experiments. On the other hand as the aggregation and sedimentation did not occur with EPC vesicles, EPA was not present in the experiments with EPC vesicles reported here. It was, however, checked that up to 10% EPA, no changes occurred in the CD spectra (data not shown). Further, a stock solution of filipin (10^{-2} mol/l) was prepared daily in DMSO and appropriate quantities of this solution were dissolved in the buffer to obtain the desired antibiotic/lipid molar ratios (we designated by r_C the filipin/cholesterol molar ratio and by r_{PL} the filipin/phospholipid molar ratio). For the incorporation of filipin during LUV preparation a filipin-buffered solution ($2 \cdot 10^{-3}$ mol/l to 10^{-2} mol/l in concentration depending on the desired proportion of the drug) was directly added with organic solvent to about 10 μmol of lipids, before sonication and reverse phase evaporation: mixed PC/cholesterol/filipin LUV (either EPC/cholesterol/filipin (85 : 10 : 5 and 65 : 3 : 30) or DMPC/cholesterol/filipin 50 : 25 : 25) were prepared according to this procedure. The lipid phosphorus content was determined as reported in Ref. 16 and the sterol content was determined by using the enzymatic test 'C system' provided by Boehringer.

CD spectra. CD spectra were recorded with a Jobin-Yvon Mark IV dichrograph with a wavelength accuracy of ±2 nm. All spectra were recorded after the samples were equilibrated at desired temperatures. Each spectrum was corrected for light scattering by vesicles. In the figures $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient ($l \cdot mol^{-1} \cdot cm^{-1}$).

Results

I. CD spectra of filipin in buffer

As already described [17,9], the spectrum of filipin in buffer is concentration dependent. Below $2 \cdot 10^{-5}$ mol/l three small negative peaks ($\Delta\varepsilon = -2$) are observed at 323, 338 and 356 nm. Above $2 \cdot 10^{-5}$ mol/l a new spectrum suddenly appears for increasing concentrations: it consists in an intense excitonic dichroic doublet centered at 295 nm with a positive band at 302 nm and a negative one at 289 nm ($\Delta\varepsilon_{302} = +13$ and $\Delta\varepsilon_{289} = -16$ for a filipin concentration of $1.2 \cdot 10^{-4}$ mol/l). This doublet is assigned to the self-association of the antibiotic. Below $2 \cdot 10^{-5}$ mol/l the antibiotic is in the monomeric form.

II. Filipin in the presence of cholesterol-free DMPC LUV at 15°C or 30°C

When DMPC LUV were progressively added to monomeric filipin (antibiotic concentration below $2 \cdot 10^{-5}$ mol/l) a new CD spectrum first appeared, identical to that already described with self-associated aqueous filipin in the presence of these LUV [9]. This spectrum consists in a characteristic doublet ('PC-type' doublet) with three positive bands at 327, 343 and 367 ± 5 nm and a negative one at 308 ± 3 (cf. Fig. 1A). The amplitude of this spectrum for increasing DMPC to filipin molar ratios was followed at 367 nm because at this wavelength monomeric filipin exhibits no CD signal. The 367 nm band height reached a maximum for a phospholipid to filipin molar ratio of approximately 4 and then progressively decreased (cf. Fig. 1B): the dichroic doublet was superseded by a weakly intense CD spectrum similar to that of monomeric filipin in buffer but slightly red-shifted (2 to 3 nm shift). The evolution was similar at 15°C and at 30°C (LUV in gel state or

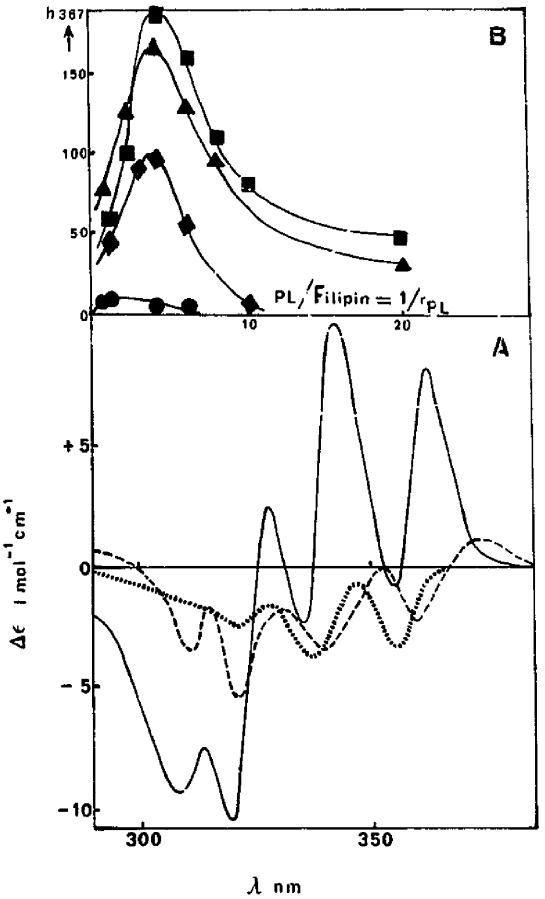

Fig. 1. (A) CD spectra of filipin (5 μM) in the presence of preformed DMPC/EPA (95:5) LUV at 15°C. ·····, [PL] = 0, r_{PL} = 0; ———, [PL] = $5 \cdot 10^{-6}$ mol/l, r_{PL} = 1; — — —, [PL] = $5 \cdot 10^{-4}$ mol/l, r_{PL} = 0.01. (B) Height of the 367 nm positive band (in mm for a light path of 1 cm and a sensitivity of 10^{-6}) as a function of the phospholipid to filipin molar ratio for the following concentrations of filipin: ●, $2 \cdot 10^{-6}$ mol/l; ◆, $5 \cdot 10^{-6}$ mol/l; ▲, $10 \cdot 10^{-6}$ mol/l; ■, $15 \cdot 10^{-6}$ mol/l.

in liquid-crystalline state, respectively) but the bands were more intense at 15°C.

III. Filipin in the presence of 25 mol% cholesterol-containing DMPC LUV

By adding increasing amounts of filipin ($0 < r_C < 10$) we observed at first, for $r_C < 1$, ([Fil] < $2 \cdot 10^{-5}$ mol/l) a modification of the spectrum of monomeric filipin with the appearance of bands already described [8] ('sterol-type bands', cf. Fig. 2A): the negative bands of free monomeric filipin were enhanced and red-shifted (at 334, 350 and 370 nm, respectively, with $\Delta\epsilon = -8$); the positive band of free filipin at 243 nm was strongly increased and a new positive band appeared at 249 nm ($\Delta\epsilon = 3$ to 11 depending on filipin concentration).

Then, for r_C values above 1, the 'PC-type' doublet described above appeared progressively characterized by its negative band at 308 nm. The relative importance of this doublet and of the 'sterol-type bands' was followed as a function of the filipin concentration at two wavelengths (Fig. 2B): at 370 nm for the 'sterol-type bands' and at 308 nm for the 'PC-type' doublet. At these wavelengths the two types of spectra do not overlap. The evolution was followed at 15, 30 and 50°C. The height of the CD signals at 370 nm reached a maximum or a plateau for r_C around 1. In contrast the height at 308 nm increased continuously for filipin concentration up to $2 \cdot 10^{-4}$ mol/l, this increase was strongly temperature dependent and favoured by a lowering of temperature.

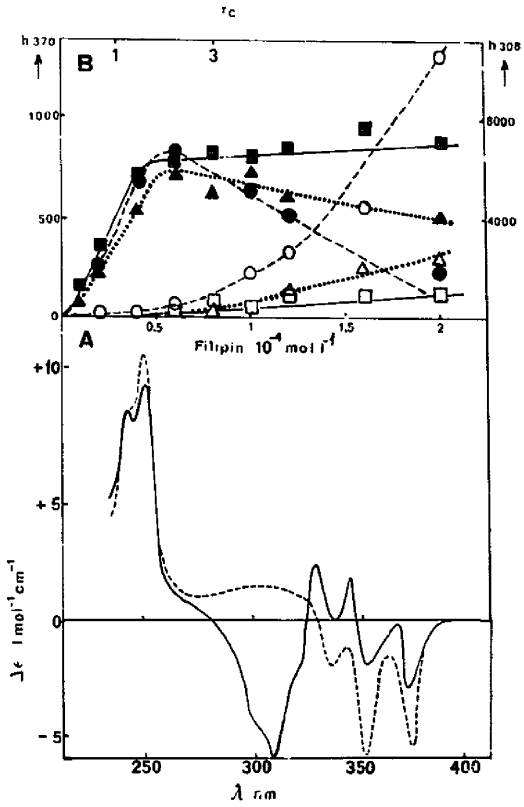

Fig. 2. (A) CD spectra of filipin in the presence of preformed DMPC/EPA/cholesterol (65:10:25) LUV at 30°C. — — —, [Fil] = $2 \cdot 10^{-5}$ mol/l, [PL] = $10 \cdot 10^{-5}$ mol/l, r_C = 0.6, r_{PL} = 0.2. ———, [Fil] = $16 \cdot 10^{-5}$ mol/l, [PL] = $10 \cdot 10^{-5}$ mol/l, r_C = 5, r_{PL} = 1.6. (B) Heights of the 370 nm and 308 nm CD bands, respectively, characteristic of filipin-cholesterol and filipin-DMPC associations □■ at 50°C, △▲ at 30°C and ○● at 15°C as a function of filipin molar ratio r_C (heights in mm for a light path of 1 cm and a sensitivity of 10^{-6}; [PL] = $10 \cdot 10^{-5}$ mol/l).

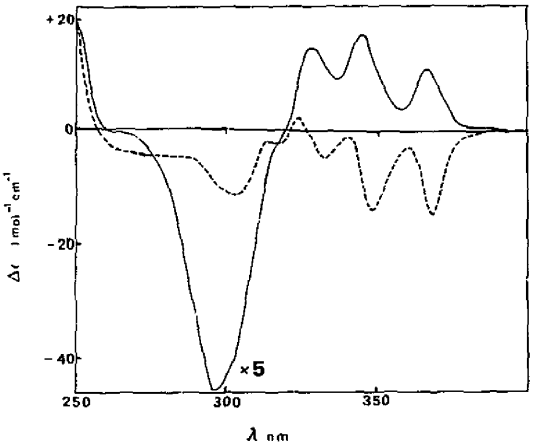

Fig. 3. CD spectra at 6 °C, of mixed LUV EPC/cholesterol/filipin with the following molar proportions: ———, 65:3:30 LUV ($r_C = 10$ and $r_{PL} = 0.5$) diluted at [PL] = $1 \cdot 10^{-3}$ mol/l. — — —, 85:10:5 LUV ($r_C = 0.5$ and $r_{PL} = 0.06$) diluted at [PL] = $1 \cdot 10^{-3}$ mol/l.

IV. Filipin incorporated during the preparation of cholesterol-containing DMPC LUV

Samples were prepared in the same conditions described by Dufourc et al. [10] for their ^{2}H-NMR study; the DMPC/cholesterol/filipin molar proportions were 6:3:3 (that is $r_C = 1$ and $r_{PL} = 0.5$). The CD spectra exhibited the 'sterol-type bands' whose intensities were divided by a factor two by increasing the temperature from 6 °C to 45 °C, in a reversible manner (data not shown).

V. Filipin incorporated during the preparation of cholesterol-containing EPC LUV

Samples were prepared in the conditions described by Maurin et al. [6] for their ESR study. As these authors, we worked at 6 °C and we used filipin molar ratios with respect to cholesterol varying by a factor twenty: either EPC/cholesterol/filipin molar proportions of 85:10:5 (that is $r_C = 0.5$ and $r_{PL} = 0.06$) or 65:3:30 (that is $r_C = 10$ and $r_{PL} = 0.5$).

For $r_C = 0.5$, we observed the 'sterol-type bands' and for $r_C = 10$, the 'PC-type' bands (cf. Fig. 3).

Discussion

Interaction of filipin with preformed pure DMPC LUV

It is interesting to compare our results with those obtained by Schwarz et al. [18] on the alamethicin interaction with dioleoylphosphatidylcholine SUV. In both studies, circular dichroism was used to monitor the conformational changes of the drug, added in the monomeric form, in the presence of increasing concentrations of phospholipid vesicles. With alamethicin, the amplitude of the CD signal characteristic of the bound species, reached a plateau for high phospholipid/alamethicin ratios but the individual curves levelled off towards markedly reduced plateau values when the alamethicin concentration was decreased. It was concluded that monomeric alamethicin, beyond a critical aqueous concentration ($2.5 \cdot 10^{-6}$ mol/l), interacts with the membrane by aggregating in the bilayer. With filipin, up to a phospholipid/filipin molar ratio of 4, we obtained similar results; the excitonic doublet observed confirms that the drug self-associates in the membranes. Above a phospholipid/filipin molar ratio of about 4, in contrast with the results obtained with alamethicin, the amplitude of the dichroic doublet decreased and a new spectrum appeared, characteristic of monomeric filipin in a hydrophobic environment. The existence of this maximum precludes to apply here the theoretical treatment developed by Schwarz et al. [18]. We shall therefore simply say, that for low phospholipid to drug molar ratios and above a critical concentration of approximately $1 \cdot 10^{-6}$ mol/l, aqueous monomeric filipin interacts with DMPC membranes and self-associates; the same interaction is observed with self-associated aqueous filipin and from the features of the ^{2}H-NMR spectra of deuteriated DMPC/filipin model membranes with molar proportions of 7:3 ($r_{PL} = 0.4$) it was concluded [9] that a filipin-phospholipid complex was formed. For high phospholipid to drug molar ratios, filipin would be bound to the membrane under a monomeric form as the CD spectrum is similar to that observed for monomeric filipin in aqueous solutions, except that it is red-shifted because of the change of polarity of the environment.

Interaction of filipin with preformed cholesterol-containing DMPC LUV

For increasing concentrations of filipin, features different from those observed with pure DMPC LUV first appeared ('sterol-type bands'). The amplitude of the 370 nm signal characteristic of these bands reached a maximum or a plateau for $r_C = 1$ (cf. Fig. 2B); consequently we assign these bands to a filipin-cholesterol complex with the stoichiometry 1:1. To compare our results to those of Bittman et al. [7] on EPC SUV, we must assume that filipin binds to cholesterol according to a mass action law expressed with volume concentrations. The association constant found is then about $4 \cdot 10^4$ M^{-1}, according to the filipin concentration at half-height of the maximum of the plateau of saturation. This value is weaker than that found with SUV ($1.25 \cdot 10^6$ M^{-1}). To compare our results to those of Maurin et al. [6] we must assume that filipin partitions first in the lipid phase, then binds to cholesterol. Furthermore the drug is assumed to be totally incorporated in the membrane. The equilibrium constant, expressing the concentrations as molar fractions with respect to total lipids, is then found to be 4. The agreement with the value given by Maurin et al., that is 6.2, is reasona-

ble. It is worthwhile to note that these bands, and therefore the filipin conformation, are the same in the presence of cholesterol-containing DMPC and EPC LUV.

Then, when the amount of filipin was increased at r_C molar ratios higher than 1, the same bands as with sterol-free DMPC LUV appeared: we deduce from this observation that the association of filipin with phospholipid could occur when all the sterolic fixation sites were occupied. The variation with the temperature of the different characteristic CD signals (cf. Fig. 2B) indicates that the formation of the filipin-cholesterol complex increased at the expense of the filipin-phospholipid complex when the temperature was enhanced from 15°C to 50°C. This is in agreement with UV absorption measurements [9] showing that the association constant of filipin with DMPC decreased when the temperature was increased from 15°C to 50°C.

Comparison of the results obtained by ^{2}H-NMR, ESR and CD on the interaction of filipin with cholesterol-containing model membranes (drug added during the preparation of membranes)

On the one hand, Maurin et al. [6] using the paramagnetic analogue of cholesterol, CNO, evidenced two kinds of spectra depending on the filipin proportions; for EPC/CNO/filipin model membranes 60:6:30 in molar proportions ($r_C = 5$ and $r_{PL} = 0.5$ in our denomination) they observed an ESR spectrum ('E type') characteristic of a spin exchange. Then, for mixtures EPC/CNO/filipin model membranes 20:1:80 in molar proportions ($r_C = 80$ and $r_{PL} = 4$) they observed an other ESR spectrum ('I type') characteristic of an immobilized form of the probe.

On the other hand, two ^{2}H-NMR studies [10,9] dealt with the action of filipin on DMPC model membranes; cholesterol was present in the first one [10] and not in the second one [9]. The first study, dealing with membranes containing equimolar proportion of cholesterol and filipin, described the effect of filipin on two view points. Using labelled cholesterol, it was shown that cholesterol sensed two environments: an immobilized site and a labile site; the authors concluded that filipin immobilized the cholesterol ring on a time scale of 10^{-5} s and thus, ESR and NMR observations are consistent with a gathering of cholesterol in a phase stabilized by filipin. On the second view point, using labelled DMPC, it could not be found, down to 5°C, any clear appearance of a gel type pattern in the NMR spectra, which led to the following conclusion: the filipin-cholesterol complex would act as a screen preventing the phospholipid from any aggregating action of filipin. However, in the second ^{2}H-NMR study, at temperatures comprised between the transtion temperature (T_c = 22°C) and 35°C the spectra of labelled DMPC evidenced two regions: one of them had the spectral features of gel-like lipids and was attributed to a filipin-phospholipid complex.

Now, let us examine our results. For cholesterol-containing membranes and $r_C \leq 1$, CD shows the same typcial spectrum in the presence of EPC (cf. Fig. 3) or DMPC (cf. Section IV, data not shown). Thus we can say that the three methods confirm the formation of a 1:1 filipin-cholesterol complex. This stoichiometry was already postulated by several workers by taking advantage of the changes of the UV [19,8] and fluorescence [20] filipin spectra in the presence of model membranes. Then, for EPC/cholesterol/filipin membranes at 6°C with an excess of filipin ($r_C = 10$, r_{PL} = 0.5, Fig. 3), CD shows a filipin–phospholipid interaction identical to the one observed for 7:3 DMPC/filipin membranes at 30°C [9] (CD spectra of preformed LUV in the same conditions for which the NMR spectra of labelled DMPC evidenced a filipin-phospholipid complex with gel-like properties). Consequently we think that the 'I-type' ESR spectrum (with $r_{PL} = 4$) corresponds to the confinement of CNO in gel-like domains corresponding to filipin-phospholipid aggregates. Furthermore complementary NMR experiments with labelled DMPC membranes containing cholesterol should evidence, for these high r_{PL}, such a filipin-DMPC aggregation, even in the presence of cholesterol.

In conclusion we showed that filipin was able to enter into a combination with two essential components of membranes, cholesterol and phospholipids. On the one hand, in the presence of cholesterol-containing model membranes, filipin in concentrations equivalent to that of cholesterol exhibited a typical CD spectrum independent of the nature of the surrounding phospholipid. On the other hand for larger filipin concentrations (equivalent to those of phospholipid) we observed an other typical CD spectrum characteristic of an aggregation of the drug (an other conformation of membrane-bound filipin); by referring to recent findings of ESR and ^{2}H-NMR we assigned the last CD spectrum to filipin-phospholipid aggregates with a gel like structure. This association with phospholipid, occurring when the sterolic sites are occupied, is strongly dependent on the temperature: it notably decreases when the temperature increases from 15°C to 50°C.

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