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Permeabilizing action of filipin III on model membranes through a filipin-phospholipid binding

Jeannine Milhaud

Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Université Pierre et Marie Curie, Paris (France)

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The binding of the pentaene antibiotic filipin to egg-yolk phosphatidylcholine (EPC) and dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles, has been studied by ultraviolet (UV) absorption and circular dichroism (CD). A stoichiometry of one molecule of filipin for five molecules of phospholipid was demonstrated by CD when phospholipids were in fluid phase. The similarity of the CD spectra with EPC and DMPC established a similar filipin-phospholipid assemblage in both membranes. We therefore postulated that filipin incorporation leads to the formation of gel-like domains in fluid EPC membranes as previously demonstrated for fluid DMPC membranes (Milhaud, J., Mazerski J., Bolard, J. and Dufourc, E.J. (1989) Eur. Biophys. J. 17, 151–158). The release of fluorescent probes (carboxyfluorescein (CF) or calcein (CC)), entrapped in EPC small unilamellar vesicles (SUV), due to the action of filipin, was followed by fluorescence and CD measurements concomitantly. The following observations were made. (1) The percentage of released probe, as a function of the filipin/phospholipid molar ratios, was the same whether or not membranes contained cholesterol. (2) The permeabilization of vesicles proceeded concomitantly with filipin-phospholipid binding while filipin-cholesterol binding leveled off. (3) The release of the content of vesicles occurred by an all-or-none mechanism leaving the depleted vesicles intact. From these observations and from the previous structural findings, a new interpretation of the action of filipin is proposed. Precluding any disruptive effect, inducement of permeability would result from the high intrinsic permeability of the interfacial region at the boundaries of the gel-like domains corresponding to the filipin-phospholipid aggregates. Additionally, we obtained the permeability coefficients for the anionic forms of CC and CF across EPC SUV, $0.6 \cdot 10^{-10}$ cm s^{-1} and $2 \cdot 10^{-10}$ cm s^{-1}, respectively, as compared to $2.5 \cdot 10^{-14}$ cm s^{-1} for the counterion Na^+ (Hauser, H, Oldani, D. and Phillips, M.C. (1973) Biochemistry 12, 4507–4517).

Introduction

In contrast to polyene antibiotics such as nystatin and amphotericin B, filipin, when interacting with sterol-containing membranes, is considered a disrupting agent rather than a channel-forming one [1]. Since 1973, filipin has been known to provoke 15–25 nm diameter hemispherical protrusions in cholesterol-containing membranes, as observed by electron microscopy [2–4]. Consequently, filipin has become a popular tool in cytochemistry for determining the sterol distribution in membranes [5–7]. The concomitant observation, of filipin-induced release of large entities such as enzymes [8] led De Kruijff and Demel [9] to propose the following model; (i) the membrane-bound cholesterol associates with filipin to give bulky complexes; (ii) these complexes, accomodated inside the hydrophobic core of the membrane, provokes its deformation until fragmentation. The first statement was supported by analytical [10] and spectroscopic [11,12] methods; so, EPR spectra exhibited by a spin-probe analogue of cholesterol, established the gathering of sterol molecules by the action of filipin, when filipin is in the same proportion as sterol, and their immobilization, when it is in the same proportion as phospholipid [11], which is in agreement with ^{2}H-NMR results [12]. The membrane fragmentation, however, is questioned by several observations. The microscopic examination of filipin-treated

Abbreviations: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; CD, circular dichroism; UV, ultraviolet; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; CF, carboxyfluorescein; CC, calcein; DMPC, dimyristoylphosphatidylcholine, EPC, egg yolk phosphatidylcholine; EPA, egg phosphatidic acid; DMSO, dimethylsulfoxide; BLM, black lipid membranes.

Correspondence: J. Milhaud, Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris Cedex 05, France.

red blood cells [13] established that their hemolysis is a purely colloidal osmotic process, inhibited by the external addition of dextran, leaving ghosts intact, even at high filipin doses (filipin/cholesterol ≈ 10). An ultrastructural examination of detergent-treated and filipin-treated vesicles from avian salt gland microsomes [14] established that filipin-treated membranes were not disrupted, even at a filipin/cholesterol molar ratio of 21; however, markers with molecular weights up to 900 could penetrate. Finally, Quigley et al. [15] did not observe any perceptible modification of the *Neurospora crassa* young hyphae plasma membranes permeabilized by filipin to nucleotides. On the other hand, Sessa and Weissmann [16], Bittman [17], and ourselves [18–20], observed that filipin also bound with some phosphatidylcholines. We showed that this binding competes with filipin-cholesterol binding [19].

In the present paper, we show that vesicles containing a fluorescent-probe are permeabilized by filipin, to the same extent, whether or not their membranes contain cholesterol. They keep their integrity despite leakage and their permeabilization correlates with the filipin-phospholipid binding as followed by circular dichroism. In the light of these results, we propose an alternative interpretation for the membrane permeabilization induced by filipin.

Materials and Methods

Materials

XI E-type EPC, EPA and DMPC were purchased from Sigma (St. Louis, MO) and used without further purification. Cholesterol was purchased from Carlo Erba and was recrystallised in ethanol before use. Filipin was purchased from Sigma and used as 75% (70% filipin III) unless otherwise mentioned (cf. elsewhere). Filipin was prepared daily as 10^{-2} M DMSO solutions (molar ratios were designated as r for filipin-to-cholesterol and R for filipin-to-phospholipid). Gramicidin was a commercial preparation from Sigma containing a mixture of gramicidins A, B and C. 5(6)-Carboxyfluorescein (CF) was supplied by Eastman-Kodak and purified according to Ralston et al. [21]. Calcein (CC) was purchased from Sigma.

Vesicles

LUV were prepared by the reverse-phase evaporation procedure [22] (buffer was 100 mM Na_2SO_4, 2 mM Na_2HPO_4 (pH 7.3) aqueous solution); with DMPC, the previously described procedure [19] was used. All LUV suspensions were sequentially extruded through polycarbonate membranes (Nuclepore) with 1 and 0.2 μm pore diameters. To incorporate filipin during DMPC LUV preparation, buffer was replaced by a 2 mM buffered-filipin solution.

SUV were prepared by sonication of a phospholipid suspension in buffer (10 mM Hepes, 150 mM NaCl (pH 7.3)) under N_2.

Fluorescent probe-loaded vesicles were prepared by replacing buffer with a 100 mM probe solution. Loaded vesicles were separated from free probe by gel permeation chromatography on Sephacryl 500 HR (1 × 20 cm column). Phospholipid assays and scattered light profiles showed that MLV were eluted in the first two fractions, while SUV were eluted in the fourth and fifth fractions. The average internal volume of SUV, determined by the released CF after lysis with Triton, is 1 l/mol EPC.

The lipid phosphorus content of vesicles of vesicles was determined as reported by Stewart [23] and their sterol content determined by using the enzymatic test 'C system' provided by Boehringer.

Fluorescence microscopic observations

A X-100 Zeiss Plan neofluar Ph objective on an Axiovert 10 inverted microscope (Zeiss), coupled to a LH 4036 camera (Lhesa) and to an image analyzing system (Biocom 200) was used. This microscope was equipped with two sets of filters which permitted both visible (excitation filter BP 450–490, emission filter LP 520) or near UV observations (excitation filter BP 365/11, emission filter LP 397).

Dynamic light scattering

A submicron particle analyzer Coultronics N_4MD was used. The size distribution profiles of the scattered intensities as well as the mean vesicle size were automatically determined by the instrument.

Spectroscopic measurements

For UV absorption, a Cary 219 spectrophotometer was used. To determine the absorbances of mixtures of filipin and EPC vesicles containing cholesterol, with $0.1 \leqslant r \leqslant 5$, we worked at a constant vesicular concentration by adding variable amounts of filipin in a concentration range where Beer's law applies, by using lightpaths between 0.005 and 10 cm.

CD spectra were recorded with a Jobin Yvon Mark IV dichrograph equipped with a thermostated cuvette-holder. Spectra were corrected for light scattering by vesicles. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient.

For fluorimetric measurements, a Jobin Yvon JY 3C spectrofluorometer, equipped with a thermostated cuvette-holder and a magnetic stirrer was used. With suspensions of CF or CC-loaded vesicles, excitation wavelength was 490 nm and emission wavelength 520 nm. The total release of the encapsulated probe was achieved by adding Triton X-100 (10% v/v) (repetitive additions of detergent assured us of complete lysis [24]). Measurements on CF-loaded EPC SUV were

carried out at 10°C due to non-negligible leakage of CF at room temperature.

Determination of the internal concentration of probe in loaded vesicles and the fraction of the released probe

The fluorescence intensity I_i emitted by a suspension of vesicles, loaded with CF or CC at the internal concentration C_i, is proportional to C_i up to a maximum, I_{max}, for C_{max} = 10 mM, from which it decreases according to $I_i = I_{max}/Q_i$ (Q_i is a coefficient which increases with C_i up to a constant value Q_{max} for $C_i \geq 100$ mM where the fluorescence levels off) [25,26].

When vesicles, with $C_i > 10$ mM, lose their whole content by the action of Triton, fluorescence increases to I_x such as:

$$I_x / I_i = Q_i C_i \cdot R = A_i$$

where the proportionality coefficient, R, which relates to the released probe concentration, depends on the phospholipid concentration (we will publish elsewhere its detailed expression). Curves in Fig. 1, showing this dependence, are used as calibration curves to determine C_i by knowing simultaneously A_i and the phospholipid concentration. With SUV at 50 μM EPC and C_0 = 100 mM, we have:

$$I_x / I_0 = Q_{max} C_0 \cdot R \approx 20 \quad (1)$$

for both probes (17.6 for CC and 22.3 for CF).

When probes, encapsulated at an initial concentration C_0 are released from vesicles until an internal concentration $C_t = C_0 - \Delta C_t$, the fluorescence exaltation from I_0 to I_t due to the released part of the probe becomes:

$$A_t = I_t / I_0 = R Q_t \cdot \Delta C_t \quad (2)$$

Under the action of filipin the fluorescence intensity at time t may be written, if f_t is the fraction of permeabilized vesicles:

$$I_t = f_t \left(\sum_i A_i I_0 + \sum_i I_i \right) + (1 - f_t) I_0 \quad (3)$$

and after lysis with Triton:

$$I_x = R Q_{max} C_0 I_0 \approx 20 I_0$$

for a 50 μM suspension.

The fraction of released probe, F_t, can correspond to two extreme cases: either every vesicle is reached by filipin ($f_t = 1$) and loses a fraction $F_t = \Delta C_t / C_0$ of its content or reached vesicles, corresponding to a fraction $f_t = F_t$ of vesicles, lose their whole contents, leading to the fluorescence intensity I_x.

In the first case, we have from Eqn. 3:

$$I_t \approx A_t I_0 = R Q_t \Delta C_t I_0$$

So, the released probe fraction $F_t = \Delta C_t / C_0$ is from Eqns. 1 and 2.

$$F_t = (I_t / I_x) \cdot (Q_{max} / Q_t) \quad (4)$$

In the second case, which we have anticipated to be the process occurring, we have from Eqn. 3:

$$I_t = F_t I_x + (1 - F_t) I_0$$

from where:

$$F_t = (I_t - I_0) / (I_x - I_0). \quad (5)$$

We calculated the percentages of released probe from Eqn. 5.

By considering the action of gramicidin, we assumed that each vesicle contained, at the concentrations used, some functional gramicidin channels [27] and, consequently, there was a progressive leak of probe from all vesicles simultaneously, according to:

$$C_t = C_0 - \Delta C_t = C_0 e^{-t}$$

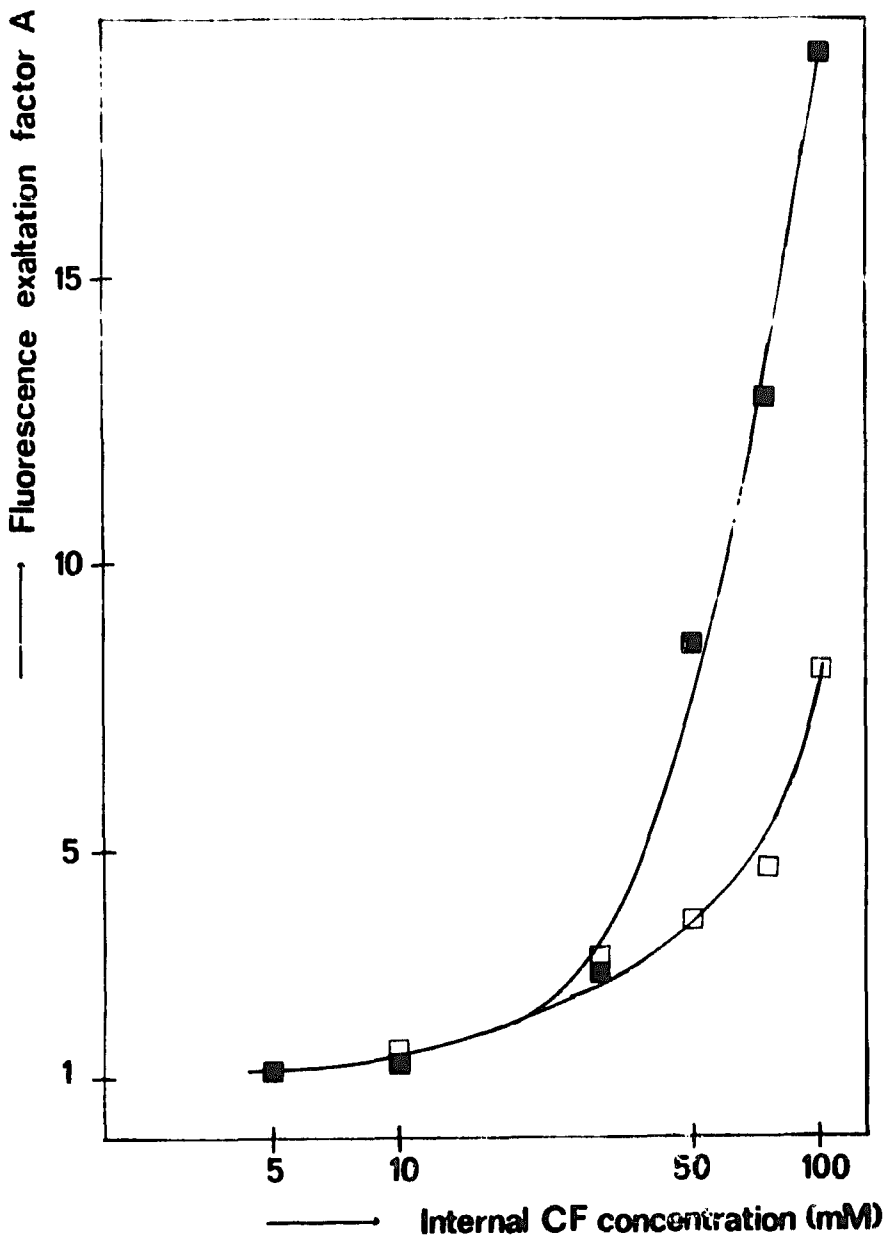


Fig. 1. Fluorescence exaltation factor, A, by lysis of CF-loaded EPC SUV by Triton, as a function of the internal CF concentration, for two phospholipid concentrations. ■, EPC = 30 μM; □, EPC = 1.5 μM.

from where the first-order rate constant k, is:

$$k = 1/t \ \ln(1/[1-(\Delta C_t / C_0)]$$

Now, from Eqn. 4:

$$\Delta C_t / C_0 = F_t = (I_t / I_\infty)_{t-0}$$

from where:

$$k = 1/t \ \ln[I_\infty/(I_\infty - I_t)]_{t-0} \qquad (6)$$

Sequential analysis of the probe content of filipin-treated vesicles

Different doses of filipin were added to aliquots of CF-loaded EPC SUV, and distributed in tubes stood at 10°C for 15 min. An excess of cholesterol (≈ 2 mM) was then added and tubes stood at room temperature for 20 min; they were then centrifugated at $10000 \times g$ for 5 min. Finally, 200 μl of each supernatant was dropped on the top of a small Sephadex G-50 column in order to separate SUV from the released CF; these columns, were placed in centrifugation tubes according to the method of Fry et al. [28]. After 10 min of centrifugation at $50 \times g$ and 3 min at $1000 \times g$, 'eluates' were collected in the bottom of tubes; they were submitted to two complementary tests: the measurement of the fluorescence exaltation factor after Triton treatment, A, and the phospholipid concentration assay. Using calibration curves (Fig. 1), the internal CF concentrations of each aliquot were then determined. By this procedure, the separation of SUV from free filipin and the released CF, was carried out, without dilution of samples, in a short time period (< 1 h) preventing any prolonged change.

Results

(A) Filipin-cholesterol binding as determined by UV absorption

The spectrum of aqueous filipin was modified by incubation with free or vesicle-bound sterols with an increase of the ratio of te absorbances at 322 and 357 nm [18]; therefore, this absorbance ratio has been empirically used to quantitate and compare the binding of filipin to different sterols [29–31]. The absorbance ratios A_{322}/A_{357} from suspensions of EPC SUV and LUV containing cholesterol, incubated with filipin, are plotted as a function of the molar ratio, r, in Fig. 2; in both cases, maxima can be seen, for r very close to 1. We interpret these maxima as follows: filipin bound to cholesterol would be characterized by an absorption spectrum with a A_{322}/A_{357} absorbance ratio higher than that of the free filipin spectrum. The superposition of two spectra would result in a A_{322}/A_{357} ratio

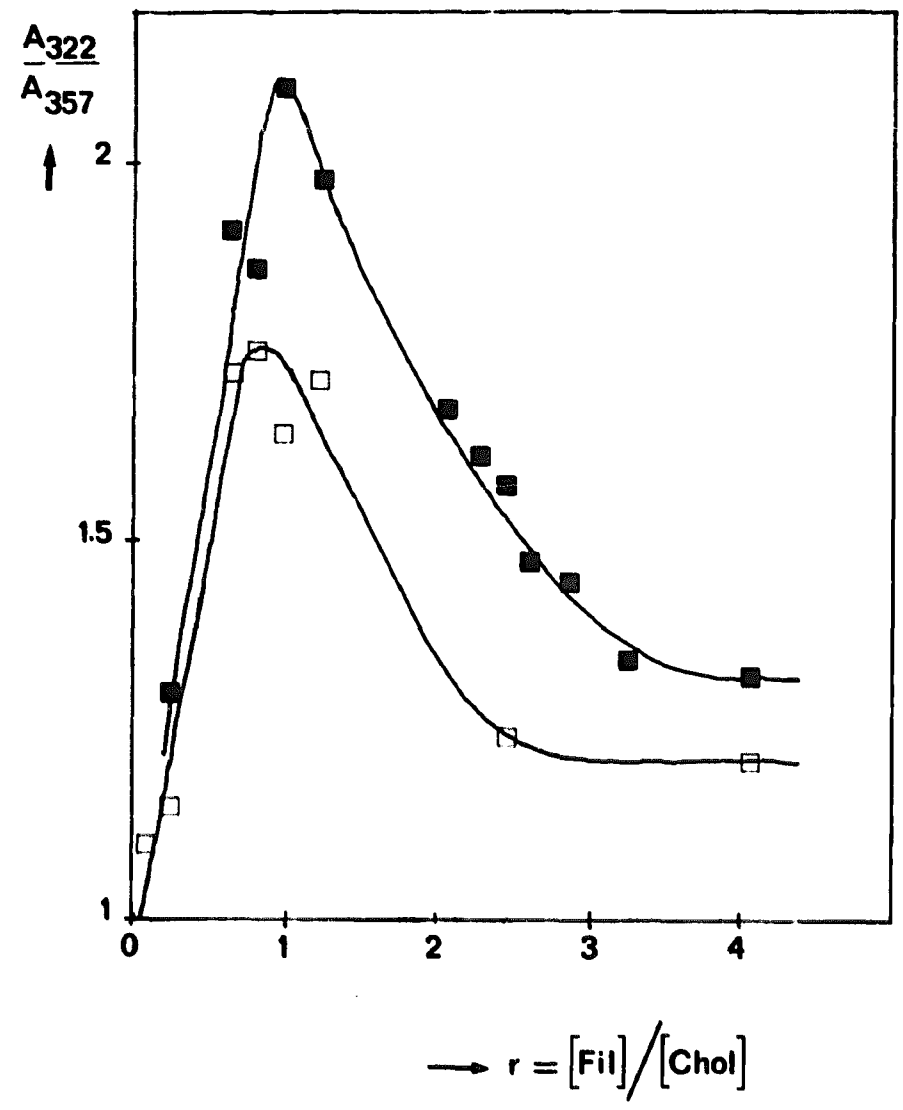


Fig. 2. Ratios of absorbances at 322 and 357 nm for suspensions of cholesterol-containing EPC vesicles (EPC = 50 μM) treated by filipin, as a function of the filipin III-to-cholesterol molar ratio r. ■, EPC/CHOL (88:12) SUV; □, EPC/CHOL (83:17) LUV.

intermediate between those of free and bound filipin. So, as r increases, the A_{322}/A_{357} ratio is smaller prior to and after completion of the filipin–cholesterol complex. Therefore, the observed maxima suggest that the stoichiometry of the filipin-cholesterol binding 1 : 1.

(B) Filipin-phospholipid binding as determined by circular dichroism

To complete previous studies [19,20] filipin was added to preformed DMPC SUV, or EPC SUV and LUV, or filipin was incorporated to DMPC LUV during their preparation. The addition to filipin of preformed fluid-phase DMPC or EPC vesicles (at 30°C and 22°C, respectively), leads to results similar to those already observed ([19] and inset of Fig. 4). The spectrum of free self-associated filipin [32], exhibiting a doublet with a null point at 295 ± 3 nm, changed into an other one which had three positive peaks at 327, 343 and 367 nm, and a null point and a negative peak red-shifted, respectively, to 313 ± 3 nm and 305 ± 3 nm. with reference to our ^{2}H-NMR results [20], this spectrum was attributed to filipin-phospholipid aggregates [19]. By varying the molar ratio, R, the intensities of the signals characteristic of this spectrum (either the negative peak or the 367 nm positive peak) went

through a maximum; this maximum can be explained in a similar manner as the maximum observed in UV absorption: the corresponding R values reflect the stoichiometry of the filipin-phospholipid binding [19]. By adding to filipin fluid-phase DMPC SUV, or EPC SUV and LUV, the stoichiometry was always one filipin molecule for five phospholipid molecules ($1/R = 5$, cf. Fig. 3).

The modification of the spectrum of DMPC LUV, where filipin was incorporated during their preparation, was followed as a function of temperature (Fig. 4). The results confirmed those of the ^{2}H-NMR experiments [20]: when the temperature increases from 32°C to 46°C, the spectrum of the phospholipid-bound filipin becomes similar to a free filipin spectrum indicating the disintegration of filipin-DMPC aggregates. Finally, owing to the important variation of the dichroic signal around 313 nm, when filipin goes from a free state to a phospholipid-bound state, Scatchard diagrams were obtained. The slopes of such diagrams are usually negative; a positive slope in the beginning of these plots, with maxima, indicates a positive cooperativity [33]. No negative slopes were obtained with fluid-phase

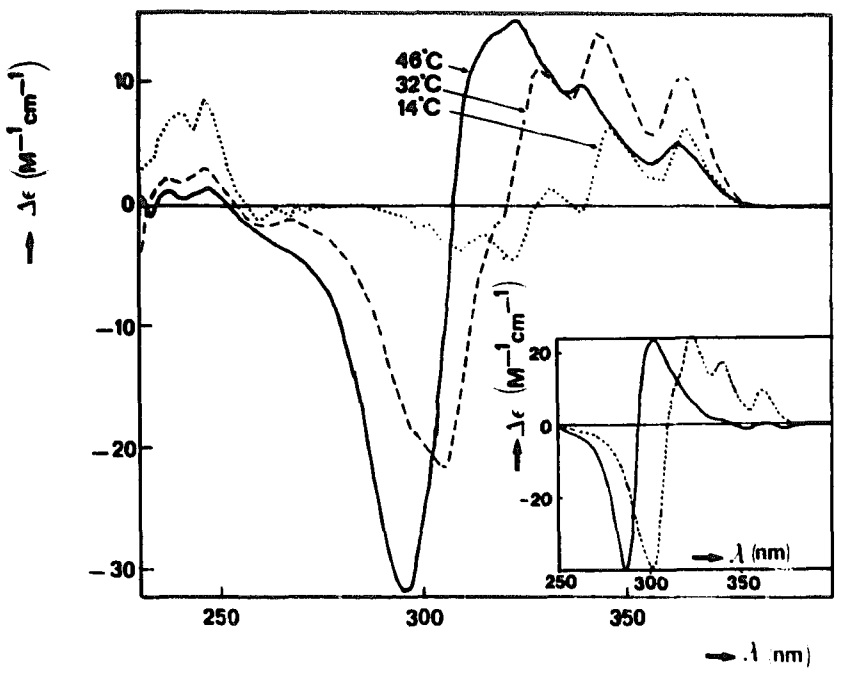


Fig. 4. CD spectra of DMPC/EPA/filipin (75:5:20) LUV as a function of temperature (DMPC = 8 mM; for preparation, cf. Methods). Inset: CD spectra of filipin (120 μM) in the presence (· · · · · ·) or absence (———) of preformed DMPC/EPA (95:5) LUV at 30°C with $R = 1$.

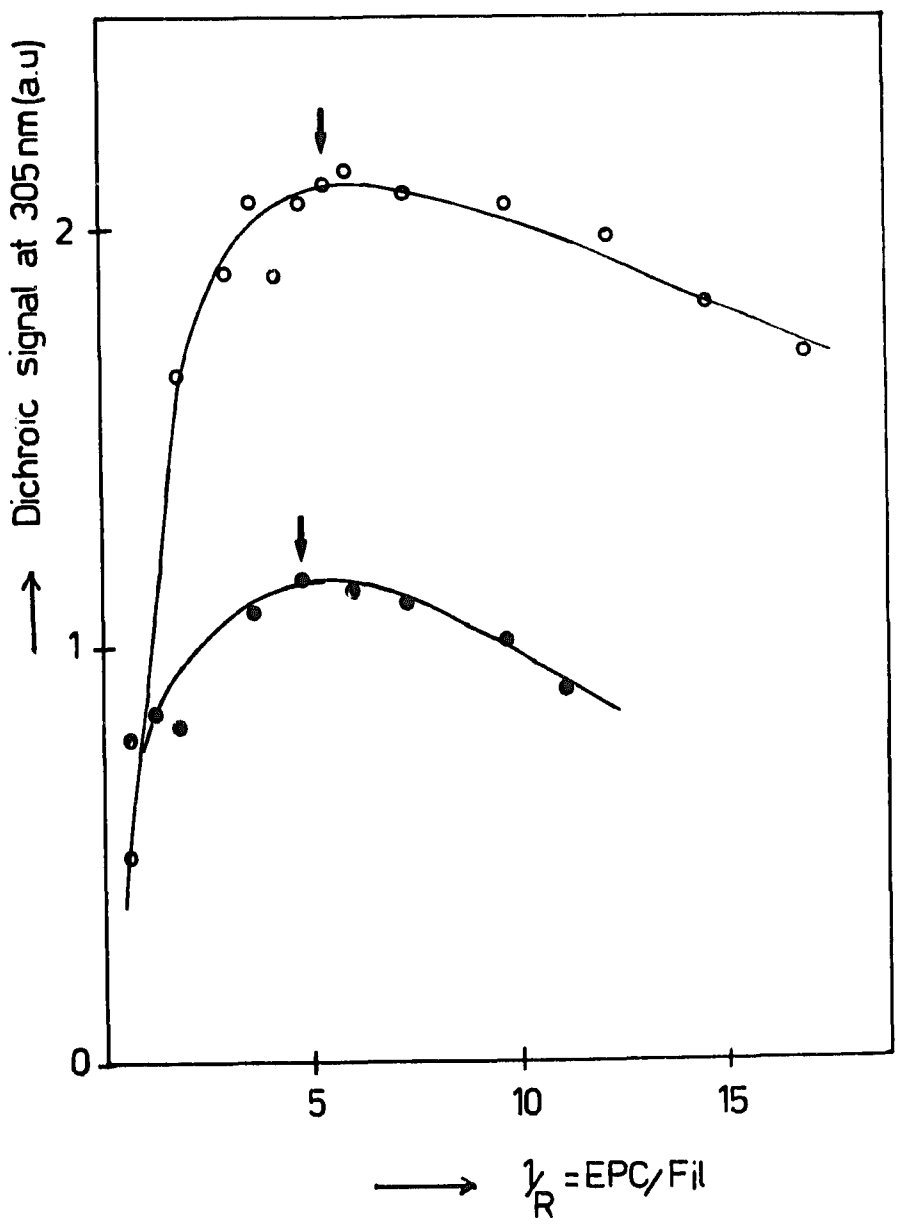


Fig. 3. 305 nm amplitudes of the dichroic signal of suspensions of cholesterol-free EPC SUV and LUV incubated with filipin (81 μM) as a function of the filipin III-to-phospholipid molar ratio R, at 22°C (incubation time: 15 min). ○, EPC LUV; ●, EPC SUV.

DMPC and EPC LUV, and SUV (data not shown). However, the first parts of these plots revealed a strong positive cooperativity in the filipin-phospholipid binding and established that filipin bound more strongly to LUV than to SUV.

(C) Fluorescence probe release from loaded vesicles

The increase of fluorescence of suspensions of EPC SUV and LUV, containing CF or CC, reflecting the probe efflux due to the action of filipin, was followed. This efflux was slow compared to the filipin-phospholipid binding followed by circular dichroism: a fluorescence plateau, was reached in 30 to 60 min with CF and 60 to 120 min with CC; it is slower with cholesterol-free membranes than with cholesterol-containing ones but the fluorescence plateau had the same intensity.

The percentage of released CF (at 10°C, cf. Materials and Methods) from EPC SUV containing CF was plotted as a function of R; experimental points met at the origin via a straight line whether or not membranes contained cholesterol (cf. Fig. 5A). It was the same for the percentage of CC released from EPC SUV containing CC at 22°C (cf. Fig. 5B). However, the percentage of CF released is larger than that of CC for the same R value. With LUV, the initial slope of the straight line is greater than that of SUV (cf. Fig. 5A). It is important to note that, for these EPC vesicles, membranes were in fluid phase.

To interpret these permeabilization results in the light of our earlier CD observations [18,19]. a dichroic spectrum of every sample of EPC SUV, containing

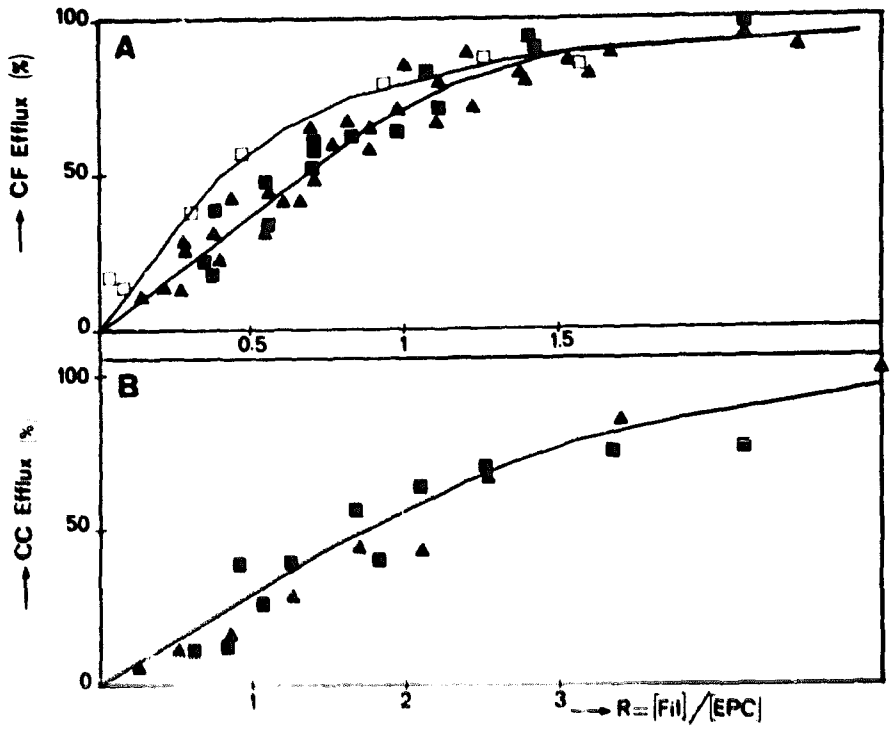


Fig. 5. Percentages of released fluorescence probe from loaded EPC vesicles as a function of the filipin III-to-EPC molar ratios. (A) Vesicles containing CF at 10°C (EPC concentration range: 20 to 300 μM). □, EPC LUV; ▲, EPC/Chol (80:20) SUV; ■, EPC SUV. (B) Vesicles containing CC at 22°C (EPC concentration range: 50 to 130 μM). ▲, EPC/Chol (80:20) SUV; ■, EPC SUV.

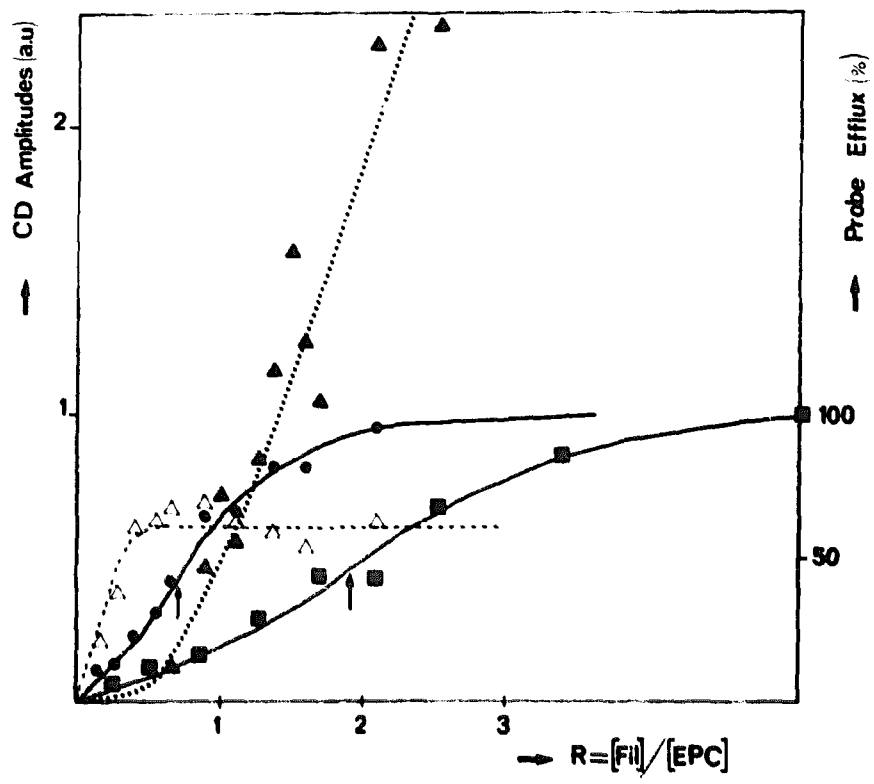


Fig. 6. 368 nm and 305 nm amplitudes of the negative dichroic signals from suspensions of EPC vesicles containing 20% of cholesterol and CF or CC, treated by filipin, as a function of filipin III-to-EPC molar ratios (at 10 and 22°C for CF and CC, respectively) and the corresponding percentages of released probe. △, 368 nm dichroic signal (corresponding to the filipin-cholesterol binding); ▲, 305 nm dichroic signal (corresponding to the filipin phospholipid binding); ●, percentage of released CF; ■, percentage of released CC.

cholesterol, loaded with CF or CC, treated by filipin, was performed before the addition of Triton to obtain 100% efflux. Results are shown in Fig. 6. The amplitudes of the negative dichroic signals characteristic of the filipin-cholesterol binding and the filipin-phospholipid binding, at 368 nm and 305 nm, respectively, showed the competitive behavior which we have already reported [18,19]. In particular, the signal characteristic of the filipin-cholesterol binding leveled off even though CF and CC continued to efflux from the vesicles (cf. Fig. 6, arrows).

TABLE I

CF internal concentrations of loaded EPC SUV aliquots after treatment by different filipin doses [a] and percentages of empty vesicles recovered after chromatography

[a] 15 min filipin treatment at 10°C; [b] EPC SUV; [c] EPC/chol 75:25 SUV; [d] under lysis by Triton X-100 of a 1.5 μM EPC aliquot after chromatographic separation; [e] for determinations cf. Materials and Methods; [f] for determinations cf. text.

Sample		Filipin (in μM)	Global CF content (in mM)	Exaltation fluorescence factor [d]	Corresponding genuine CF content [e] (in mM)	Empty vesicles (%) [f]
	0	0	100			
I [b]	1	160	82	7	92	11
	2	370	13	8	100	87
	0	0	100	5		
II [b]	3	160	67	6	83	20
	4	370	48	10	100	52
	0	0	100			
III [c]	5	107	54	5	70	13
	0	0	100			
	6	97	95	9	100	5
IV [c]	7	160	58	8	100	42
	8	240	30	7	92	67
	9	370	13	5	70	81

Sequential analysis of CF content of filipin-treated EPC SUV

To analyse the internal CF concentrations of aliquots of EPC SUV containing CF, treated by different filipin concentrations, the released probe was separated after treatment from the recovered SUV (cf. Materials and Methods). Our initial aim was to establish if the probe efflux results from a complete release from a part of vesicles or a partial release from all vesicles simultaneously. In the first case, the probe concentration of remaining loaded vesicles will stay unchanged while, in the second one, it will decrease by increasing the filipin dose (cf. Weinstein et al. [34] and Blumenthal et al. [35]). However, the crucial point, here, is to demonstrate that vesicles, partially or totally depleted, keep their integrity. Results of 4 batches of EPC SUV are presented in the Table I; two of them contained 25 mole% of cholesterol. The third column gives the average CF content of SUV after treatment, as determined by the fluorescence after lysis with Triton, by assuming that all vesicles are identical (progressive CF efflux from all vesicles simultaneously). The fifth column gives the actual CF content of SUV as deduced from the fluorescence exaltation after triton treatment given in the fourth column (cf. Materials and Methods). These actual concentrations clearly disagree with the average ones: they remain constant around 85 ± 15 mM and it can be therefore concluded that the mechanism was all-or-none. In corollary, it is easy to calculate that an important portion of recovered vesicles were empty. Indeed, fluorescence intensity after lysis divided by the total number of vesicles, or by the number of loaded vesicles, were respectively proportional to the CF concentrations given in the third and the fifth columns: so, the complement to 1 of the ratio of these two concentrations gives the proportion of empty vesicles; the corresponding percentages are given in the sixth column. Thus, depleted vesicles were chromatographically eluted; they keep the integrity of their membranes whether or not they contain cholesterol.

A significant percentage of vesicles, were not recovered when a cholesterol treatment, before chromatography, was not performed (cf. Materials and Methods). This observation agrees with Kelly et al. [10] who found that the recovery percentage of EPC vesicles, submitted to a prolongated incubation with filipin, followed by gel filtration on Sepharose 4B, was small. We attributed this fact to their agglutination as described below.

(D) Miscellaneous investigations

(1) Agglutination of vesicles by the action of filipin

Suspensions of cholesterol-free EPC LUV containing CF or CC (at 10 mM concentration), were observed by fluorescence microscopy before and after filipin treatment. Before treatment, uniformly scattered bright points were observed; after 45 (CF) or 75 (CC) min of treatment, these points clustered together, here and there, inside a veil which was visible by excitation in near UV, where only filipin absorbs.

In addition, the granulometric behavior of suspensions of cholesterol-free EPC SUV without probe, after filipin treatment, was followed by light scattering. The scattered light profile of the initial suspension, as a function of particle size, exhibited a major peak corresponding to SUV (30 nm diameter) with a much smaller peak corresponding to residual MLV after chromatography. After incubation with filipin at $R = 0.5$, a new peak, corresponding to clusters with diameters approximately 300-times larger than those of SUV, appeared. We checked that a filipin solution at the same concentration showed no such cluster. After addition of an excess of cholesterol (cholesterol/filipin $\approx$ 4) to dissolve clusters the suspensions became turbid and, after centrifugation (5 min at 15000 rpm), the scattered light profile of supernatant showed a reappearance of SUV, with a disappearance of filipin–SUV clusters.

From these observations we conclude that the vesicles clustered together with filipin. This phenomenon was already remarked by Behnke et al. [4].

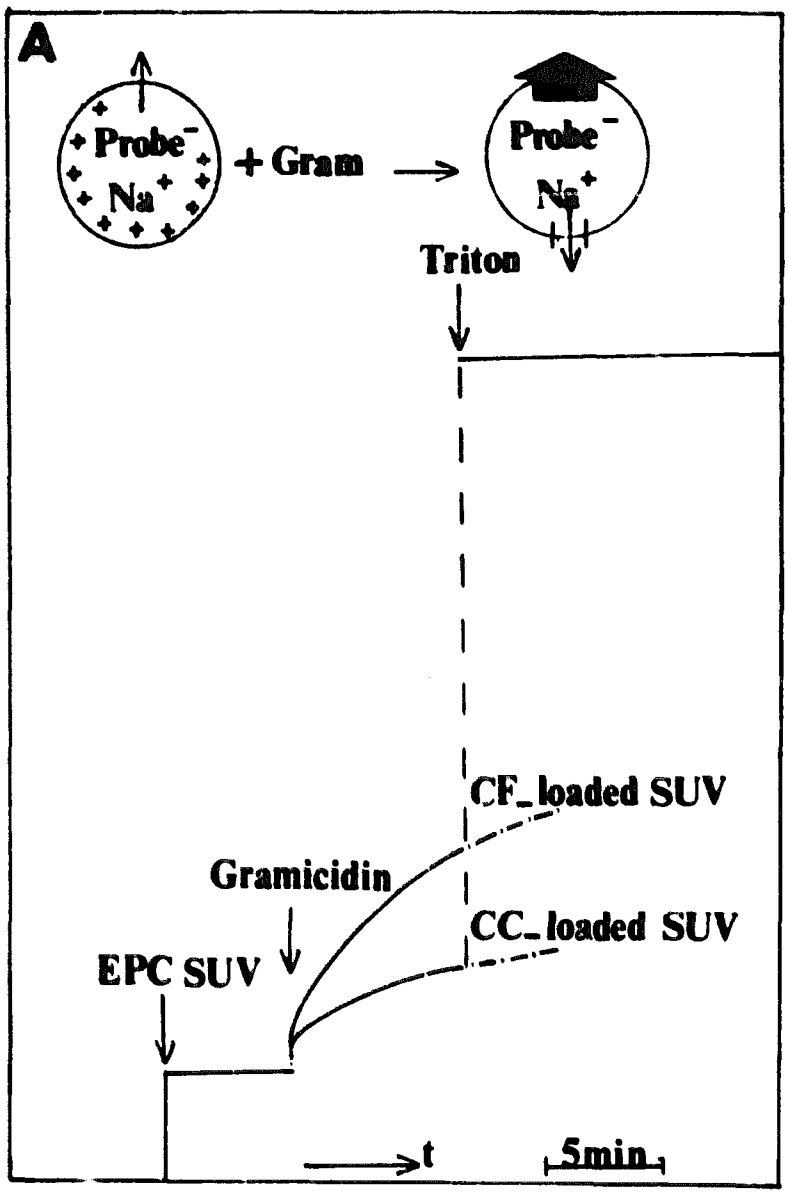
(2) Permeabilizing action of gramicidin: 'natural' translocation rates of CF and CC

Bramhall et al. [36,37] demonstrated that the anionic probe CF released freely from unilamellar vesicles; its release was simply prevented by the membrane potential developed by the absence of a concomitant release of the alkaline counterion. By establishing a free circulation of this last one, owing to the addition of an ionophore such as gramicidin, it is therefore possible to assess the 'natural' translocation rates of CF and CC, what we did in order to interpret the curves of Fig. 5; 10 μM of gramicidin was added to 50 μM of CF- or CC-containing EPC SUV and the fluorescence increase followed (cf Fig. 7A). The corresponding first order rate constants for the release of the two probes (for calculations see Materials and Methods) are reported in Fig. 7B. These rate constants can easily be transformed to permeability coefficients, P, by knowing the trapped volume, V (1 l/mol of EPC, cf. Materials and Methods) and the corresponding surface area, A, of the membrane system (65 Å^2/mol of EPC, cf. Ref. 38):

$$P = k \cdot V / A = 2 \cdot 10^{-10} \text{ cm s}^{-1} \text{ for CF}$$

$$P = 0.6 \cdot 10^{-10} \text{ cm s}^{-1} \text{ for CC.}$$

These numbers correspond to the permeabilities of anions; they are, respectively, two and four orders of



B	CF (10°C) (1)	CF (10°C) (2)	CC (22°C) (1)
K(s^{-1})	8.2×10^{-4}	6.2×10^{-4}	2.4×10^{-4}
P (cms^{-1})	2×10^{-10}	1.5×10^{-10}	0.6×10^{-10}

(1) cholesterol_free SUV

(2) cholesterol_containing SUV (EPC/Chol 90/10)

Fig. 7. (A) Action of 10 μM gramicidin on suspensions of EPC SUV containing CF or CC ([EPC] = 50 μM); (I_∞ corresponds to 100% release, and is achieved by adding Triton). (B) The corresponding first-order rate constants and permeability coefficients for the CF and the CC release, respectively.

magnitude larger than Na^+ permeability coefficients through EPC LUV ($9.5 \cdot 10^{-13}$ cm s^{-1} cf. Ref. 39) and EPC SUV ($2.5 \cdot 10^{-14}$ cm s^{-1} cf. Ref. 40). This confirms Deamer and Bramhall's general statement [41] that, for model membranes, anion permeability is significantly greater than that of cations.

(E) Influence of the filipin composition on its permeabilizing action

The filipin complex contains four components labelled I, II, III and IV [42] where III and IV are the two isomeric forms [43,44] responsible for the fungicidal activity of the filipin complex [42]. However, in most reports, the major component of filipin complex has not been specified. For example 95% 'pure' filipin as determined by UV absorption [8] may refer to filipin complex (53% filipin III cf. Ref. 42), or to filipin III alone, since, both products exhibit approximately the same absorption spectra [45]. In 1986, Filipin complex was first commercially provided as 40% (w/w), and later, as 75% (w/w, 70% filipin III); pure filipin III has been available since 1990. Most of our experiments were performed with filipin '75%'. However, some of experiments, were repeated using the pure filipin III. Our results referred to the portion of the filipin III deduced from the extinction coefficient ($\varepsilon_{345} = 9.2 \cdot 10^4$ mol^{-1} cm^{-1} in DMSO), were in satisfactory agreement with those obtained with pure filipin III. In contrast, we were not able to reproduce with filipin '40%' the results obtained with filipin '75%'. Indeed, with filipin '40%', we found results identical to those described by Maurin et al. [46], characterized by a 10-times higher permeabilizing action of filipin and a strong dependence on the presence of cholesterol in membranes. In conclusion, our permeabilizing results, were assignable to filipin III but not those obtained with filipin '40%'.

Discussion

(1) Previous results

^{2}H-NMR spectra of cholesterol-free DMPC membranes, in which 12% filipin was incorporated during their preparation, revealed the presence of gel-like domains enclosed in the bulk fluid; their proportion decreased as temperature increased. They were attributed to filipin-DMPC aggregates [20].

Dichroïc measurements of filipin incubated with preformed cholesterol-free DMPC LUV revealed the aforesaid spectrum [19,20] assigned to the filipin-DMPC aggregates. When DMPC LUV containing cholesterol were treated by increasing doses of filipin a spectrum first appeared [19] which had been previously observed for SPC LUV containing other β-hydroxysterols and attributed to a filipin-sterol complex [18]. For larger amounts of filipin, this spectrum was replaced by the one obtained with cholesterol-free DMPC LUV [19].

(2) Structural results (Figs. 2 and 3):

The stoichiometry for the two filipin binding was established. Filipin–cholesterol has a 1:1 stoichiometry (Fig. 2), confirming Schroeder et al. [47] and Katzenstein et al. [48]. For filipin-EPC binding, a 1:5 stoichiometry was established (Fig. 3) at concentrations of self-associated filipin (> 20 μM), as previously obtained for filipin-DMPC [19]. Furthermore, Scatchard's plots revealed positive cooperativity in filipin-phospho-

lipid binding. Thus, the ordered packing of phospholipids by the action of filipin, as showed by ^{2}H-NMR of DMPC membranes [20], occurred by cooperative types of interactive forces.

Thus filipin binds to cholesterol-free EPC and DMPC membranes. The CD spectra of self-associated filipin interacting with both types of membranes in fluid phase were identical, indicating that this interaction leads to molecular assemblages with the same conformation for both phospholipids. We infer that the filipin incorporation into the fluid EPC membranes leads to the formation of gel-like domains, as observed with fluid DMPC. Since filipin is a neutral molecule, we think it anchors in the hydrophobic part of membranes; we suggest that macrolide rings are extended along acyl chains and to optimize the Van der Waals interaction, we think this is their polyene portions which would be close to them, while hydrophilic portions would be gathered together, forming an hydrophilic core. The occurrence of such ordered assemblages inside the fluid bulk of membranes would give at each leaflet the aspect of a mosaïc (cf. Fig. 8A). The implication of such an organization is discussed below.

(3) Permeabilization results (Figs. 5 and 6, table I):

Bramhall et al. [36,37] clearly demonstrated that CF, and consequently CC, are released freely through membranes in their anionic forms and that the step which controls their efflux is the release of the alkaline counterion, i.e. Na^+. Our experiments in the presence of gramicidin confirm this. The 'natural' efflux rate of CF through EPC SUV is about three times larger than this of CC (Fig. 7B). Filipin apparently acts like gramicidin; in agreement with a CF efflux three times faster than the CC one, the release percentage of CF is about three times larger than that of CC for the same filipin/EPC molar ratio (Figs. 5A and 5B). Consequently, we will discuss only of a Na^+ permeabilizing action of filipin.

Does the probe efflux occur by a progressive mechanism on all vesicles or by an all-or-none mechanism on a fraction of them? Let us do a general remark about the action of a permeabilizing agent on a suspension of loaded vesicles. Let us suppose that the permeabilization of one vesicle requires the gathering of a certain number of agent molecules inside its membrane to 'open a channel'. Because of the distribution of membranes in separated closed units, the release of the vesicle contents implies a competition between two kinetic processes (i) the exchange of the permeabilizing agent between vesicles, (ii) the depletion of one vesicle during the opening time of one 'channel'. Two extreme cases can be discriminated. If the rate of process (ii) is much higher than that of process (i) the depletion occurs by an 'all-or-none' mechanism where a portion of vesicles lose the whole of their contents. If the inverse is true, the depletion occurs by a 'progressive' mechanism where all vesicles are simultaneously permeabilized by losing a portion of their contents. In the present case, the efflux rates of both fluorescent probes, CF and CC, were small (Fig. 7B). Nevertheless, the positive cooperativity of the filipin-phospholipid binding and the persistent agglutination of a vesicle fraction under the action of filipin, suggested that the redistribution of filipin among vesicles was slower than this efflux. Therefore, the release mechanism should be 'all-or-none'. As a matter of fact, this is confirmed by the analysis of the CF content of filipin-treated EPC SUV (Table I).

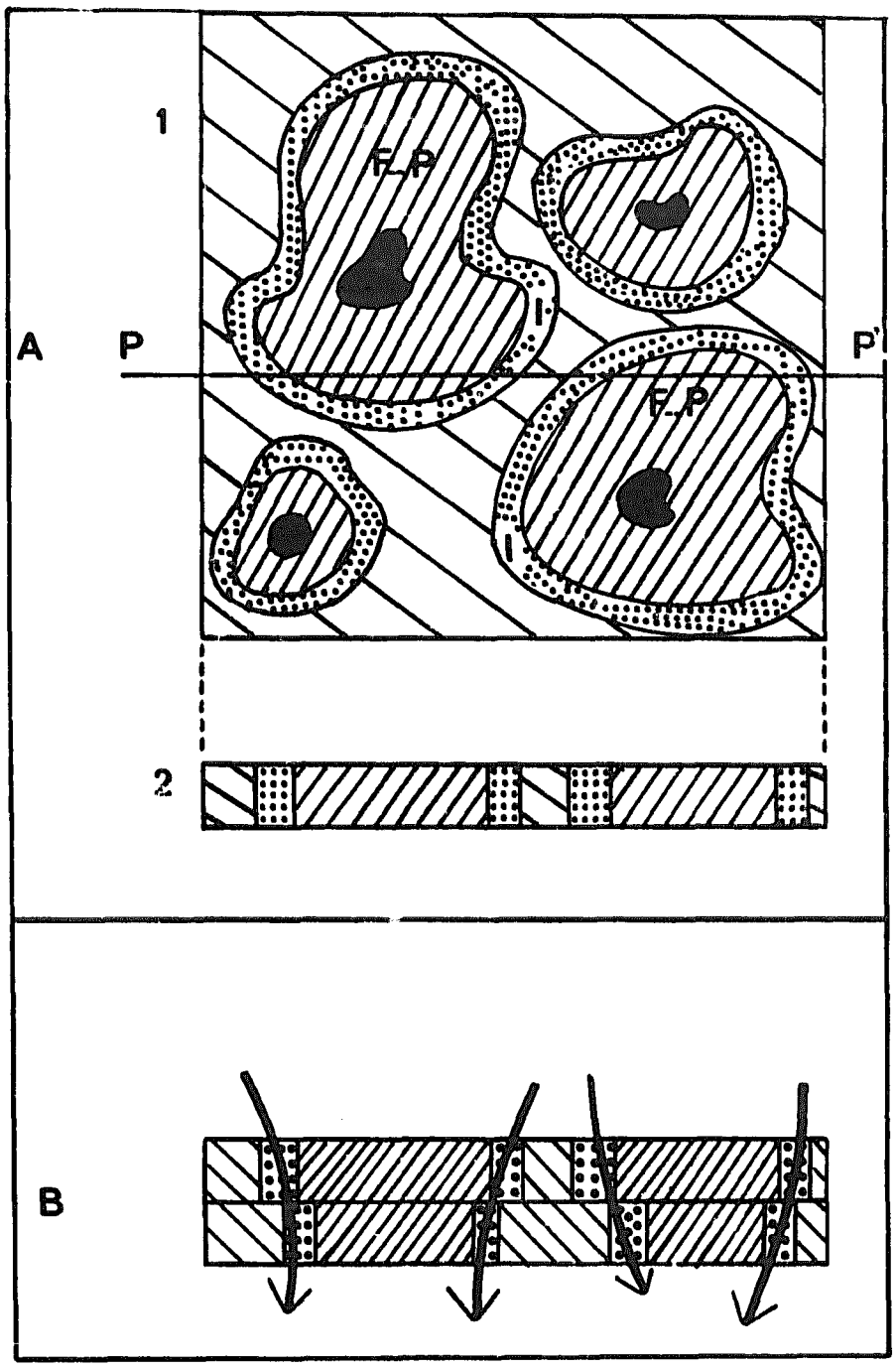


Fig. 8. (A) Tentative illustration of the lateral configuration at the surface of a leaflet of a bilayer by the action of filipin; 1: Top view; 2: cross section PP'(F-P: ordered phospholipid; I: interstitial region). (B) Cross section of a bilayer with 'open channels'.

Now, let us discuss De Kruijff and Demel's model [9] assuming a disrupting action of filipin in the presence of cholesterol. Sequential analysis of CF content of EPC SUV clearly show that depleted vesicles were not destroyed whether or not their membranes contained cholesterol since empty like full vesicles were chromatographically separated from free probe (Table I).

Two additional arguments allow us to reject any disruptive action of filipin on membranes: one, the prolonged (several hours) observation of filipin-treated loaded EPC LUV, by fluorescence microscopy; secondly, the ^{2}H-NMR spectra exhibited by the filipin-treated DMPC membranes preclude any destabilization of them even in the presence of cholesterol [49]: especially, they are in any case assignable to mixed micelles (Dufourc, E.J., private communication).

What is then the permeabilizing mechanism, especially in the absence of cholesterol? Dichroic spectra performed during the permeabilization followed by fluorescence showed (Fig. 6) that the efflux of both probes occurred in parallel with filipin-phospholipid binding while the filipin-cholesterol binding had already levelled off. Thus, the filipin-phospholipid binding can be held, responsible for the permeabilizing action of filipin. Now, Deamer and Bramhall recently analysed [41] mechanisms which make lipid bilayers permeable to hydrophilic solutes. They emphasized that there is significant penetration of surrounding water into bilayers (see Refs. 50 and 51). Thus, an hydrophilic solute can penetrate as far as the hydrophobic part of a membrane, anchor, if a defect can accomodate it, and, from there, migrate by solubility-diffusion. The presence and the number of defects depend on the physical state of the membrane (lipid composition, temperature, presence of extraneous agents). 'Transitory defects', providing some sites of entry would increase in number when interactive strengths of cooperative type, act on the hydrocarbon chain packing, such as gel-to-fluid transitions or embedding of extraneous agents. So, Marsh et al. [52] linked the enhanced tempocholine release from loaded DMPC SUV at the phase transition temperature to the coexistence of domains of gel and fluid phases by inferring that, at the interface of these domains, occur interstitial lipids whose the intrinsic permeability would be larger than that of membrane bulk. This interpretation allows to explain similar phenomena: with pure phospholipids, the occurrence of maxima for the permeability of anions or cations at the phase transition temperature [53,37] and, with mixtures of phospholipids, the same at temperatures corresponding to the coexistence of two phases [54,55]. It is in line with a recent modelling [56] which links the enhanced passive permeability of lipid bilayers at the transition temperature to the lateral heterogeneity resulting from the cooperative fluctuations of lipid chain conformations. We will use the same interpretation to account for the action of filipin. We think that an increased Na^+ permeability, leading to a parallel CF or CC release from loaded vesicles, may occur at the edges of the ordered domains corresponding to filipin-phospholipid aggregates. We failed to visualize appearence of defects in freeze-fractured DMPC MLV, with filipin embedded during the preparation of membranes, at temperatures where ^{2}H-NMR spectra showed the appearance of gel-like domains. However, we are not concerned with domains of different symmetry, only distinguishable: we consequently think this absence of visibility does not affect the credibility of our thesis. Let us emphasize that the segregation process of phospholipids around filipin, leading to a mosaic-like organization, would occur independently on both leaflets of bilayers. Consequently, it is only when the two mosaic-like leaflets would have their interstitial phospholipid annulii overlapping, that there would be an 'opening of a channel', as visualized in the Fig. 8B.

Finally, is the permeabilizing mechanism the same in the absence and in the presence of cholesterol? Two arguments prompt us to think this: (i) the CD spectra of filipin-treated loaded EPC SUV clearly show that the filipin-cholesterol binding has levelled off before the complete development of permeabilization (Fig. 6); (ii) the CF and CC efflux percentages as a function of filipin/EPC are the same (Figs. 5A and 5B) whether or not vesicles contain cholesterol. However, a recent paper [57] has shown that the conductance increase of mixed 1-palmitoyl,2-oleoylphosphatidylethanolamine and 1-palmitoyl,2-oleoylphosphatidylcholine (the main component of EPC) BLM, by the action of filipin, occurred only in the presence of cholesterol. We think that this disagreement results from the difference in the membrane systems used. It is well known that SUV have special properties due to their high curvature [58,59].

In conclusion, we established that the first stage for permeabilization of SUV by the action of filipin corresponded to their agglutination, with loss of vesicular contents, without damaging the membranes. Consequently, the protrusions observed by electron microscopy on cholesterol-containing membranes treated by filipin (equally apparent with pure filipin III and with filipin 40%, Dunia, I and Benedetti, J., private communication), corresponding to the gathering [11] and the immobilization [12] of membrane cholesterol, does not provoke the membrane disruption. We can tentatively visualize these protrusions as a core of cholesterol covered by a filipin mantle, itself surrounded by phospholipids ordered under its influence, when we deal with DMPC and EPC bilayers. So, we reject our previous proposal [20] and we dismiss the 'screen' model proposed by Dufourc et al. [60] and Dufourc [61] which suggested that cholesterol would prevent filipin from direct interaction with phospholipids. The depletion of vesicles would occur owing to 'defects' (not visualizable as mechanical fractures but rather like annulii of interstitial phospholipid with an increased intrinsic permeability), at the edges of ordered phospholipid domains. Although, in this first stage, membranes stay intact we do not exclude that,

with prolongated incubations with filipin, vesicles can fuse with loss of their integrity as it appears with other membrane perturbants like melittin [62].

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